

Scanning Probe Recognition Microscopy Investigation of the Elastic Properties of Tissue Scaffolding

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ABSTRACT

Scanning Probe Recognition Microscopy is a new scanning probe capability under development within our group to reliably return to and directly interact with a specific nanoscale feature of interest, without the use of a zoom box with its thermal drift and local origin difficulties. It is a recognition-driven and learning approach, made possible through combining SPM piezoelectric implementation with on-line image processing and dynamically adaptive learning algorithms. Segmentation plus a recognized pattern is implemented within a scan plan and used to guide the tip in a recognition-driven return to a specific site.

The specific application focus of our group is on the development of Scanning Probe Recognition Microscopy for nanobiological investigations. In the present work, Scanning Probe Recognition Microscopy is used in a direct investigation of the surface and elastic properties along individual tubules within a tissue scaffolding matrix. Elastic properties are indicated as important influences on actin polymerization and consequent cell pseudopodia extension and contraction.

INTRODUCTION

Scanning probe recognition microscopy is a new scanning probe capability under development within our group to reliably return to and directly interact with a specific nanoscale feature of interest, without the use of a zoom box with its thermal drift and local origin difficulties. It is a recognition-driven and learning approach, made possible through combining SPM piezoelectric implementation with on-line image processing and dynamically adaptive learning algorithms. Segmentation plus a recognized pattern is implemented within a scan plan and used to guide the tip in a recognition-driven return to a specific site.

The specific application focus of our group is on the development of Scanning probe recognition microscopy for nanobiological investigations. In previous work, we have successfully recognized and classified tubular versus globular biological objects from experimental atomic force microscope (AFM) images using a method based on normalized central moments^{1,2}. Normalized central moments are translation, rotation and scale invariant. We have also extended this work to include recognition schemes appropriate for more subtle differences between biological objects of similar globular external boundaries with dissimilar internal features by adding the Continuous Wavelet Transform (CWT) with a differential Gaussian mother wavelet³. The 2-D continuous wavelet transform allows multi-scale analysis of images. Thus, these two methods together can be applied to analyze biological objects of any scale.

In the present work, scanning probe recognition microscopy is used in a direct investigation of the surface and elastic properties along individual tubules within a tissue scaffolding matrix. Elastic properties are indicated as important influences on actin polymerization and consequent cell pseudopodia extension and contraction. It is therefore important to analyze the elastic properties of tissue scaffolds to find the best match for specific cell types.

METHODS

Atomic force microscopy (AFM) can be used to measure elastic properties by collecting force curves over points on the surface of sample^{4,5,6}. A single force curve records the force felt by the tip as the sample and tip are approached and retracted. This is done by recording the cantilever deflection as a function of distance between the tip and the sample surface. It is more useful to collect arrays of force curves across the sample surface at regular intervals and this is known as force volume imaging⁷. A force volume data set can be used to generate a 3-D map of interaction forces between a sample and the tip.

In ordinary force volume imaging, a height image of the sample surface is simultaneously captured. In Scanning Probe Recognition Microscopy, we first analyze this height image to recognize the scaffold locations that we are interested in. We then combine this recognition with AFM force volume imaging of the elasticity of just the tissue scaffolding. This is a more targeted and more computationally efficient method of elasticity analysis.

Recognition

We first use thresholding methods to identify and discriminate the tissue scaffold from the background pixels. Since there are also some high amplitude noise pixels present, two threshold values are designated. A high threshold value removes the noise pixels and a second low threshold value eliminates the background pixels. The thresholding filter can be expressed as

$$g(x, y) = \begin{cases} 1 & \text{if } t_1 \leq f(x, y) \leq t_2 \\ 0 & \text{otherwise} \end{cases} \quad (1)$$

where $f(x, y)$ is the original image, t_1 is the low threshold, t_2 is the high threshold, and $g(x, y)$ is the image after thresholding.

Typically the thresholds t_1 and t_2 are selected using established histogram based procedure⁸. However, thresholding alone is insufficient to eliminate all of the noise and background pixels. After thresholding, the data is passed through a median filter⁹ where the area of each connected pixel set is calculated, and those having small area are assumed to be noise pixels and removed. Finally, a morphological erosion operation is used to eliminate the boundary pixels of the tissue scaffold and to guarantee that all the pixels left are on the tissue scaffold.

Elasticity measurement

The Force Integration to Equal Limits (FIEL) mapping method is used to produce a robust measurement of relative elasticity. This method has the advantage of being independent of the tip-sample contact point, and of not requiring calibration of the AFM cantilever spring force constant.

Force curves record the cantilever deflection (d) versus the height position of the sample (Z). The Force Distance (FD) curve is defined by using an absolute distance ($D = Z - d$), which is the separation between the tip and the sample surface, instead of using sample position (Z). According to the Hertz model, if the tip of AFM is approximated as a sphere, then the force on the cantilever (F) can be calculated by

$$F = \frac{4E\sqrt{R}}{3(1-\nu)} \delta^{3/2} \quad (2)$$

where E is the elastic modulus, R is the radius of the probe sphere, δ is the indentation and ν is the poisson ratio.

The relationship between elasticity and FD curves at two positions derived from FIEL mapping method is

$$\frac{w_1}{w_2} = \left(\frac{k_1}{k_2} \right)^{2/3} \quad (3)$$

where $k = \frac{1-\nu}{\pi E}$ is elastic constant, $w = \int_0^\delta F d\delta$ is the work done by the cantilever, which equal to the area under the FD curve. The area under the FD curve can be calculated and used to represent the elasticity feature of the tissue scaffolding.

EXPERIMENTAL RESULTS

Data Acquisition

Our data was acquired using a Digital Instruments Nanoscope IIIa MultiMode Scanning Probe Microscope system. The Atomic Force Microscope (AFM) head model MMAFM 2/850EX and the 125x125 micron maximum scan range J-scanner were used. Force volume imaging, using contact mode AFM with silicon nitride probes with a nominal tip radius of 25 nm, was performed in ambient air. The cantilever spring constant was $k=0.58$ N/m. Relative triggering was used to capture the force volume data sets. 64×64 force curves were recorded over a 5×5 micron area of tissue scaffolding, with each curve (extending and retracting) sampled 64 times.

The raw data was exported from the captured force volume images as an ASCII file. Every parameter under the user's control is written into the file header and all the data is stored in the file. Matlab was then used to read the ASCII file and reconstruct the original image. All the following processing is based on the raw data from the exported file. A reconstructed height image and an example of a force curve at one point are shown in Figure 1a and 1b.

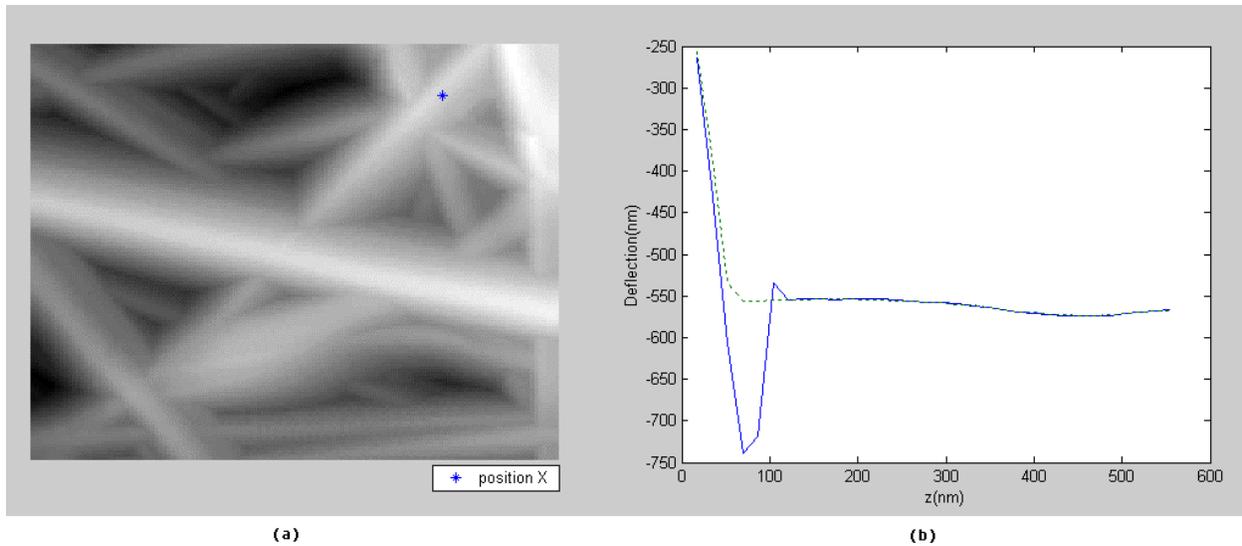


Figure 1. Reconstructed images of tissue scaffolding (a) height image, (b) force curve at position X.

Recognition

From Figure 1a, we can see that there are some extremely bright regions which are artifacts in the image that do not contain any real information. In order to eliminate those pixels and locate the pixels on the tissue scaffolding, the two-level thresholding described above was used, with the result shown in Figure 2a. The choice of the two threshold values was based on a histogram of the pixel intensities in the image. Here, the low threshold and the high threshold are chosen as 1580 and 4081 respectively. Then the small area regions were removed using a median filter and the corresponding result is shown in Figure 2b. Due to tip-rounding artifacts at the edges of the tubules, the data in the center of each tubule is the most reliable. The erosion operation was used to eliminate pixels on the boundary, as shown in Figure 2c. With these three steps, it could be guaranteed that all the chosen pixels were on the center part of a tissue scaffold tubule where the information was captured most accurately.

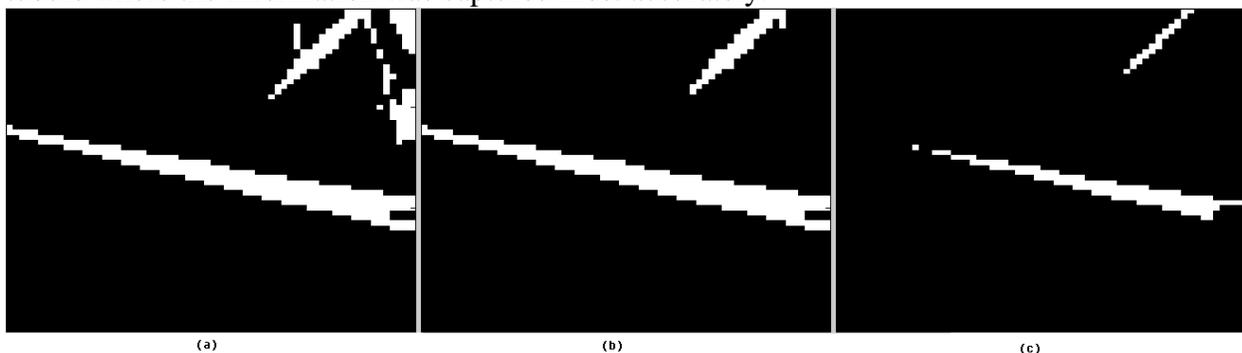


Figure 2. Detecting the locations of the center parts of tissue scaffold tubules (a) after thresholding, (b) after denoising, (c) after the erosion operation.

Elasticity

The elasticity analysis is based on the extending traces of the force curves. The retracting traces have sharp upward rebounds that correspond to surface adhesive forces, as shown in

Figure 1b, which is a different physical phenomenon than the elasticity. In the FIEL method, the force-distance curves are calculated first at all the points of the center part of a tissue scaffold tubule. One example of an extending-trace force curve and its corresponding force-distance curve is shown in Figure 3.

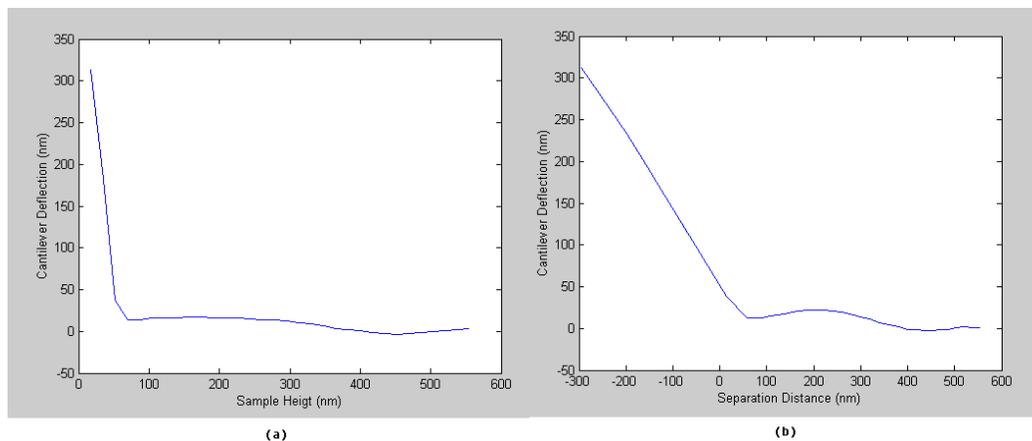


Figure 3. Extending force curve (a) force height curve, (b) force distance curve

The elastic property of the tissue can be represented by the area under the force-distance (FD) curve. For the point shown on Figure 3, the area under the FD curve was $59707 \text{ (nm}^2\text{)}$. For a background pixel, the area was $48584 \text{ (nm}^2\text{)}$ using the same method. By comparison, we can see that the elasticity of the point on the tissue scaffold was much larger than that of the background point. We calculate the areas of all these points on the reliable center parts of the tissue scaffold and use these values to create an elasticity map of the scaffold, as shown in Figure 4. We can also use statistical data, e.g. the mean value, of these areas to represent the elasticity of one tissue scaffold for comparison with another.

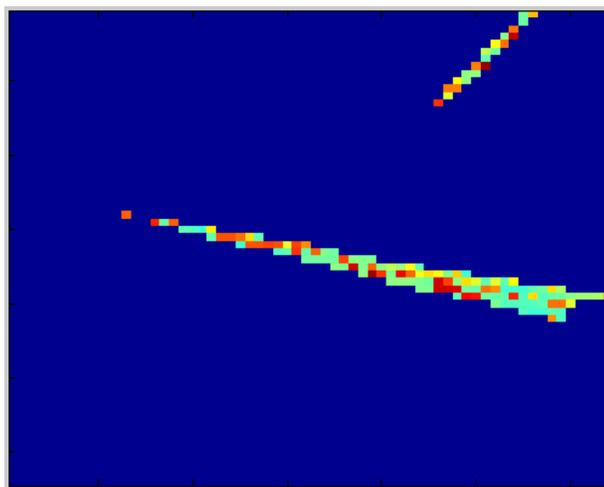


Figure 4. Elasticity map along center parts of the scaffold

DISCUSSION

This paper analyzes the elasticity of a tissue scaffold based on AFM force volume imaging. Recognition methods are first used to identify only the scaffold from the background. A simple

edge detection technique based on a two-step threshold analysis is presented here. Since the force volume data sets at the center of a tissue scaffold tubule represent the most reliable information with the least tip-rounding artifacts, erosion of the tissue scaffold boundary is also performed to isolate these most reliable points. The FIEL method is then used on only these points to calculate the elasticity of tissue scaffolding. Thus, the elastic properties of tissue scaffold can be analyzed automatically without the influence of the background and artifact-prone boundary pixels. Because it is unnecessary to map the elasticity of background and boundary pixels, the computation of this method is more efficient than the elasticity mapping of the whole force volume image.

This recognition-assisted elasticity analysis provides the capability to compare the elasticity of tissue scaffold at different locations by recognizing locations first. It could also be used to compare the elasticity of different types of tissue scaffold if the data sets are captured under same circumstances. If the elastic properties are consistent along each type of tissue, the elasticity can be extracted as a feature and used in classification of different types of tissue scaffold. Because of its efficiency, this recognition-assisted elasticity analysis method is especially suitable in on-line elasticity analysis or comparison research.

REFERENCES

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1. Q. Chen, V. Ayres and L. Udpa, "Biological Investigation Using Scanning Probe Recognition Microscopy", Proceedings 3rd IEEE Conference on Nanotechnology, vol. 2, p 863-865 (2003).
 2. Q. Chen, L. Udpa, M. S. Schindler, C. Berger and V. M. Ayres, "Scanning Probe Recognition Microscopy Investigation of Cells on Scaffolding", Mat. Res. Soc. Symp. Proc. Vol. EXS-1: *Architecture and Applications of Biomaterials and Biomolecular Materials*, ISBN: 1-55899-745-8, Ed. A. Plant, A. Chilkoti, A.E. Barron and J. Wong, 2004, The Materials Research Society, Warrendale, PA (2004), pp. 143-145.
 3. B. Goolsby, Q. Chen, L. Udpa, Y. Fan, R. Samona, B. Bhooravan, F. M. Salam, D. H. Wang, and V. M. Ayres, "Scanning Probe Microscopy with Landmark Referenced Control For Direct Biological Investigations", J. Nanosci. Nanotech., vol. 3, no. 4, p347-350 (2003).
 4. E. A-Hassan, W.F. Heinz, M.D. Antonik, N.P. D'Costa, S. Nagaswaran, C.A. Schoenenberger, and J.H. Hoh "Relative Micro-Elastic Mapping of Living Cells by Atomic Force Microscopy" Biophysical Journal, vol. 74, no. 3, p 1564-1578 (1998).
 5. Radmacher M., "Measuring the elastic properties of biological samples with the AFM", IEEE Eng Med Biol, vol. 16, p47-57 (1997).
 6. H. Haga, S. Sasaki, K. Kawabata, E. Ito, T. Ushiki, T. Sambongi, Ultramicroscopy, "Elasticity mapping of living fibroblasts by AFM and immunofluorescence observation of the cytoskeleton", vol. 82, p253-25 (2000).
 7. J.H. Hoh, W.F. Heinz, E. A-Hassan "Force Volume" Support Note No. 240 Digital Instruments. (1997)
 8. William K. Pratt, *Digital Image Processing*, Wiley-Interscience Publication, Second Edition, CA (1991).
 9. R.C. Gonzales and R.E. Woods, *Digital Image Processing*, Addison-Welsley, (1993)