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A Semi-Automated Positioning System for contact-mode Atomic Force Microscopy (AFM)

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Abstract

Contact mode Atomic Force Microscopy (CM-AFM) is popularly used by the biophysics community to study mechanical properties of cells cultured in petri dishes, or tissue sections fixed on microscope slides. While cells are fairly easy to locate, sampling in spatially heterogeneous tissue specimens is laborious and time-consuming at higher magnifications. Furthermore, tissue registration across multiple magnifications for AFM-based experiments is a challenging problem, suggesting the need to automate the process of AFM indentation on tissue. In this work, we have developed an image-guided micropositioning system to align the AFM probe and human breast-tissue cores in an automated manner across multiple magnifications. Our setup improves efficiency of the AFM indentation experiments considerably.

Note to Practitioners: Human breast tissue is by nature heterogeneous, and in the samples we studied, epithelial tissue is formed by groups of functional breast epithelial cells that are surrounded by stromal tissue in a complex intertwined way. Therefore sampling a specific cell type on an unstained specimen is very difficult. To aid us, we use digital stained images of the same tissue annotated by a certified pathologist to identify the region of interest (ROI) at a coarse magnification and an image-guided positioning system to place the unstained tissue near the AFM probe tip. Using our setup, we could considerably reduce AFM operating time and we believe that our setup is a viable supplement to commercial AFM stages with limited X-Y range.

Keywords

Atomic Force Microscopy; Biomechanics

I. Introduction

Recent research has shown that several cellular processes can be uniquely phenotyped by their mechanical signatures, e.g. proliferation of disease in cells and tissue [1] and differentiation patterns in embryonic stem cells [2]. Several methods have been proposed to study the mechanical properties of biomaterials such as micropipette aspiration, laser-based tweezers and magnetic probes [3]. However, Atomic Force Microscopy, which uses piezo-actuators for closed-loop positioning, has proved to be more reliable compared to other methods due to its superior precision capabilities and the requirement of minimal sample preparation [4].

Though piezo-actuators produce high-resolution force-spectroscopy, they are severely limited by their range of travel. Conventional AFM stages have around $100\text{ }\mu\text{m}$ range for closed-loop positioning. While this may be sufficient for analyzing biomaterials at the cellular scale, such stages do not produce closed-loop positioning of larger specimens such as histological tissue sections [5].

The study of histopathological tissue specimens fixed on a microscope slide has several advantages over phenotyping isolated cell populations. It provides direct insight into the latent architectural changes that takes place within a developing tumor and its neighboring tissue throughout the course of disease progression. Furthermore, the use of Tissue Microarray (TMA) technology allows high-throughput quantitative assessments of the changes in biomarker expression signature in normal and malignant tissue based on the staining protocols used [5] and can be validated against AFM based measurements [6].

To ensure that the malignancy of interest has been sufficiently represented on a tissue specimen, tissue cores are typically sectioned at 0.6 mm diameter, around six times larger than the range provided by commercial AFM stages. Also, a given type of malignancy might be distributed amongst multiple cores on a slide, and therefore the slide containing the tissue cores (see Fig. 1) needs to be traversed for AFM analysis on individual cores. Moreover, it becomes necessary to zoom in on a ROI to locate malignancies desired to be phenotyped and then revert back to a coarse magnification to get a larger perspective of the alignment of the ROI and the AFM probe. Such tiresome protocol repeated many times over could lead to operator fatigue [7] and hence there is a need for increased automation aid in tissue registration across multiple magnifications and to navigate through the tissue being studied.

In this work, we have addressed this problem by developing an automated image-guided positioning system to align the AFM probe and the tissue specimen across multiple magnifications, thereby eliminating the need to use manual positioning screws at the base of the AFM stage. We have used normalized cross-correlation based tracking algorithm [8] to track the tissue at coarse and fine magnifications, following which the AFM indentation experiments are carried out. Using our positioning system, we can characterize a total of 480 points in a region approximately $80\text{ }\mu\text{m} \times 150\text{ }\mu\text{m}$ in about 80 minutes; whereas performing AFM indentations using a manual positioning system on the same region would take anywhere between 2-4 hours, depending on the proficiency of the AFM operator.

The format of this paper is given as follows. In section II, we discuss the design of the TMA and the positioning system. In section III, we discuss the tracking and control scheme employed. We finally conclude with our results in section IV and discussion in section V.

II. Materials and Methods

A. Tissue Microarray Preparation and Annotation

Tissue cores of 0.6 mm diameter were removed from paraffin-embedded cancer and normal breast tissue blocks and assembled into 4 quadrupled tissue microarray blocks using Auto Tissue Arrayer (Beecher ATA-27). Two consecutive slices of each TMA, 4 μm apart, were sectioned and fixed onto glass slides. The center-to-center distance between adjacent cores on the slide was approximately 1.25 mm. A representative image of a TMA is shown in Fig. 1.

Hematoxylin and Eosin (H&E) was used for staining each set of consecutive slides. The stained slides were then cover-slipped. We used whole-slide, Virtual Microscopy (VM) technology to obtain digital scans of the tissue segments. The use of VM allows automatic scanning of a specimen at a fixed high resolution and therefore allows users to maneuver across the digital specimen as if they were examining the specimen using an optical microscope. Both the unstained and stained tissue slides were digitized into a tiled TIFF format at 40 $\times$ equivalent resolution using Trestle/Zeiss MedMicro scanning system and uploaded onto the web server at <http://virtualscope.umdj.edu> for further viewing and annotation.

The H&E slides are inspected by a pathologist to confirm the specimen's histological validity and one pair of consecutive slides containing significant amount of breast parenchyma was selected for the experiments. Following this, the pathologist then annotated valid normal and cancerous regions in epithelial and stromal tissue using the online annotation tool, while these labels were concealed to the individuals who subsequently conducted the AFM experiment. The annotated regions on the unstained slide were then probed by the AFM in a raster fashion, and the elasticity results were correlated with the pathologists' assessments of the annotated regions [6].

B. AFM Experimental Setup

Before starting the AFM experiment, the unstained slide adjacent to the annotated one was deparaffinized with xylenes and hydrated with graded alcohols and then kept in Phosphate buffered saline (PBS).

The AFM system comprises of the AFM scanning head and the controller (MFP-3D-BIOTM, Asylum Research, Inc.) coupled to an inverted microscope (Model: TE2000U, Nikon, Inc.) such that the AFM head rests on the microscope (Fig. 2). The whole setup is enclosed within an acoustic hood to isolate it from external noise. A CCD camera (QImaging Inc, Model: Retiga 2000R) is mounted to the microscope for visual servoing. The range of the X and Y-axes of the piezo-actuated stage is 90 μm and the customized range for the Z-axis is 40 μm . Situated at the base of the microscope is a motorized MP-285 micromanipulator (manufactured by Sutter Instruments, Novato, CA), to which is attached a custom-made end-effector, to which the slide is attached. The MP-285 has a step resolution of 40 nm and a range of 2.54 cm on both X and Y axes, large enough to account for multiple tissue cores on the same slide. The micromanipulator, microscope and the AFM head are placed on a vibration isolation table (manufactured by Herzan) to eliminate base vibrations. A detailed explanation of AFM probe-sample interactions is given in [2].

Since the forces measured during AFM indentation experiments are in the nm-range, chatter in the end-effector or the slide can lead to incorrect mechanical property estimation and can potentially damage the AFM probe and the tissue sample. To eliminate vibrations in the slide, the end-effector was fabricated using Aluminum and the slide is clamped to the end-effector using a clamping screw (Fig. 2 inset). Additionally, an adhesive tape was used to

firmly attach the slide to the end-effector. We found that using this arrangement, there were no perceptible vibrations in the slide/end-effector, as mentioned in Section III.

To ensure that the tissue cores are hydrated during AFM experiments, the periphery of the slide was coated with a hydrophobic barrier using a Pap-Pen. PBS solution was added intermittently to prevent the tissue slide from dehydrating. After identifying each core on the slide, the annotated ROI within each core was located by carefully following the annotated map [Fig. 1(c)] and the corresponding ROI on the tissue slide was selected for AFM probing.

III. Image-Guided Navigation

A. Tracking of the ROI

The presence of vision in the loop allows the AFM operator to select a certain ROI and place the ROI underneath the AFM probe tip. Some of the popular tracking algorithms are gradient-based methods like the Kanade-Lucas-Tomasi (KLT) feature tracker [9] and the Scale-Invariant Feature Transform (SIFT) [10]. However, as seen in Fig. 1(d), the unstained tissue images are characterized by low contrast and therefore unique feature vectors are difficult to compute. In our experience, features of high contrast (extracted from the images) have been external particles in the fluid environment, which tend to move around randomly during the motion of the tissue slide underneath the AFM probe.

As a result, we used normalized cross-correlation based template matching algorithm [8] to ensure robustness of the tracking algorithm during the motion of the slide. Though the applicability of such methods are conditional upon uniform scene lighting and in-plane translation without rotation, we could ensure these conditions by: (1) manually adjusting the external lighting and (2) ensuring that there is no relative motion in the various interconnected parts of the end-effector.

The implementation of the tracking algorithm is given as follows:

For an image, $I_t = \{I(p_x, p_y, t) | 0 \leq p_x \leq R, 0 \leq p_y \leq C\}$, and a ROI, $T = \{T(p_x, p_y, t) | 0 \leq p_x \leq r, 0 \leq p_y \leq c\}$, where I_t and T are the image and the ROI respectively at the t^{th} frame, the estimated position of the ROI at the $t + 1^{th}$ frame is given by [11]:

$$(\hat{p}_{x,t+1}, \hat{p}_{y,t+1}) = \arg \max_{p_x, p_y} \frac{\sum_{p'_x, p'_y} [T(p'_x, p'_y) I_{t+1}(p_x + p'_x, p_y + p'_y)]}{\sqrt{\sum_{p'_x, p'_y} T(p'_x, p'_y)^2 \sum_{p'_x, p'_y} I_{t+1}(p_x + p'_x, p_y + p'_y)^2}} \quad (1)$$

The numerator of the expression in (1) indicates the correlation of the ROI and the image, while the denominator is the normalizing term to ensure that general lighting differences in both the ROI and the image do not affect the tracking algorithm significantly. The tracking algorithm was implemented in Visual C++ using OpenCV libraries [11].

B. Control Law

The control law in discrete state space form is given by:

$$\mathbf{X}(k+1) = \mathbf{X}(k) + \mathbf{u}(k) \quad (2)$$

$$\mathbf{Y}(k) = \mathbf{S}\mathbf{R}\mathbf{X}(k) \quad (3)$$

where

$$\mathbf{S} = \begin{bmatrix} s_x & 0 \\ 0 & s_y \end{bmatrix}; \quad \mathbf{R} = \begin{bmatrix} \cos(\theta_0) & -\sin(\theta_0) \\ \sin(\theta_0) & \cos(\theta_0) \end{bmatrix} \quad (4)$$

$\mathbf{Y} = [p_x \ p_y]^T$ and $\mathbf{X} = [x \ y]^T$ represent the image and manipulator frame coordinates respectively. $\mathbf{R}$ is the rotation matrix between the manipulator and the image frame while $\mathbf{S}$ is a scaling matrix relating the two frames. The parameters of $\mathbf{R}$ and $\mathbf{S}$ are estimated in a pre-experiment calibration step using a cover-slip with uniform grids marked at $100 \mu m$ separation. $\mathbf{u}(k)$ is the control input in the manipulator frame. To estimate the control input, we used a gradient-descent based approach [12], where the control input $\mathbf{u}(k)$ is based on the gradient of the function:

$$F[\mathbf{X}(k)] = [\mathbf{X}(k) - \mathbf{X}_{tip}]^T [\mathbf{X}(k) - \mathbf{X}_{tip}] \quad (5)$$

The control input $\mathbf{u}(k)$ is therefore given as:

$$\mathbf{u}(k) = -\gamma(k) \nabla F[\mathbf{X}(k)] = -2\gamma(k) [\mathbf{X}(k) - \mathbf{X}_{tip}] \quad (6)$$

where the step size $\gamma(k)$ is given by:

$$\gamma(k) = \begin{cases} \frac{\gamma_0}{2\|\mathbf{X}(k) - \mathbf{X}_{tip}\|}, & \text{if } \|\mathbf{X}(k) - \mathbf{X}_{tip}\| > \varepsilon \\ 0, & \text{if } \|\mathbf{X}(k) - \mathbf{X}_{tip}\| \leq \varepsilon. \end{cases} \quad (7)$$

γ_0 is a constant that determines the magnitude of the incremental travel of the ROI towards the probe tip $\mathbf{X}_{tip}$. The positioning is terminated once the ROI is within a radius of ε from the probe tip, where ε is a preset parameter.

It is vital to ensure that parts of the tracked ROI are not occluded by the AFM probe during positioning since the template matching algorithm requires the entire ROI to be visible in the image space. As a result, the estimated position of the slide in image coordinates, $\mathbf{Y}$, is selected to lie on the upper-corner of the leading edge of the ROI, as shown in Fig. 3(a).

IV. Results

Using the tracking algorithm and the control law described in Section III, the following protocol is implemented for alignment of the tissue ROI and the AFM probe tip (see Fig. 3).

- A coarse ROI is selected at low magnification ($m_1 = 10\times$), after visually correlating the annotated regions from the stained image [Fig. 1(c)] and the brightfield microscope image from the CCD camera attached to the AFM microscope. The AFM probe tip is also selected visually at the same magnification [Fig. 3(a)].
- Based on the tracking algorithm and control law discussed, the ROI is positioned within an error of ε , chosen to be $2 \mu m$ [Fig. 3(b)].
- The objectives, lighting and focusing are altered manually at higher magnification ($m_2 = 20\times$) to ensure that the probe tip and the ROI is in focus and the scene is uniformly lit. The AFM tip is then selected visually by the user at high magnification, m_2 . Since the probe tip is stationary, it serves as a reference for the

coarse ROI, which is recreated at a distance m_2 / m_1 from the tip at m_2 magnification [Fig. 3(c)]. This allows registration of the same ROI across multiple magnifications. At m_2 magnification, finer details are visible to the user and a part of the recreated ROI is selected, called the Probing ROI, which is probed by the AFM. The remaining part of the recreated ROI serves as the Tracking ROI. The Probing ROI is then sampled in a raster fashion, while the Tracking ROI is used to provide image feedback [Fig. 3(c)].

The probing force was held at 120 nN (producing an indentation = 150nm–500nm) for a sample of $4\mu\text{m}$ thickness. A rectangular-shaped Aluminum-coated silicon probe (Novascan Inc, Ames, IA, spring constant = 4.5 N/m) with an attached glass bead microsphere ($R_s = 2.5\mu\text{m}$) was used to indent the sample. The Probing ROI (approximately $80\mu\text{m} \times 150\mu\text{m}$ in size) was sampled at $5\mu\text{m}$ intervals and the Hertz model [2] was used to generate the elasticity map of 480 points shown in Fig. 3(d). The sampled tissue shows heterogeneous stiffness ($\sim 50 - 1200$ kPa).

A representative AFM force curve obtained from samples mounted on our positioning system is shown in Fig. 3(e). Minimal fluctuations in the deflection data is indicative of negligible vibrations introduced due to the end-effector design.

The positioning accuracy is demonstrated in Fig. 3(f), where the slide is translated from its original position (262,226) to $Y_{tip} = (163, 362)$ in the image space. The positioning errors at the completion of alignment at $10\times$ and $20\times$ were $1.6\mu\text{m}$ and $0.8\mu\text{m}$ respectively, which are acceptable alignments errors in our case.

V. Discussion

In this work, we report on a reliable means for addressing low throughput in AFM indentation experiments on tissue arising due to: (1) lack of automated specimen registration across multiple magnifications and (2) positioning large tissue specimens underneath AFM probes prior to AFM indentation. We used normalized cross-correlation based template matching [8] and a micromanipulator to position the target ROI underneath the preselected AFM tip across multiple magnifications.

In the future, we will apply our setup to characterize malignancy in multiple tissue cores in an automated manner. In addition, the applicability of automated multimodal registration between stained tissue images and the unstained brightfield images from the AFM microscope will be investigated. Such improvements could potentially further reduce human intervention and lead to a greater efficiency in AFM experimental procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. An example of one tissue sample as has been used in different steps of experiment: (a) A low resolution stained image of the tissue microarray. (b) The VM image of an H&E stained tissue core. (c) The same core annotated by a pathologist. (d) Image taken from the AFM microscope during the experiment.

Fig. 2.
AFM Experimental Setup with the MP-285 micromanipulator.

Fig. 3. (a) - (c) Tracking protocol to position tissue underneath the AFM probe tip, (d) Elasticity map of the probing ROI (e) Representative AFM force curve on a sample mounted on the end-effector and (f) Tracking Performance.