

# Imaging of in Vitro Collagen Fibers by Atomic Force Microscopy

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## **Abstract**

The aim of this research was to investigate the use of atomic force microscopy (AFM) for the high-resolution imaging of collagen fibers in aqueous media. Collagen plays a vital role in many processes in vivo, especially in structural integrity, yet few studies have used AFM to investigate the structure of these fibers. Type I collagen was used in this study on AP-mica, poly-L-lysine-coated glass slides and on ethanol-cleaned glass slides to determine what structures these fibers assume in vitro under the various conditions. It was found that AFM is in fact a viable method for determining collagen structure, and that type I fibrils can be seen in both cross-linked and isolated forms. A resolution of about 20–30 nm was achieved. This research forms a foundation for further investigation into the different types of collagen structures in vivo and the environmental conditions that cause them.

## **Introduction**

Our bodies are made up of around 70% water. Why do our bodies seem so firm if we are composed of mostly fluids?

The answer comes in the form of a large network of structural support, and one substance plays a surprisingly large role in this network. As one pharmaceutical company states, “Collagen is the tie that binds the animal kingdom together.”<sup>1</sup>

Collagen is the most abundant protein found in mammals. It is the major component of the extracellular matrix, a network of cross-linked fibers surrounding cells and in the interstitial spaces between them. It comprises almost 30% of our total protein mass and is one of the primary components of major tissues such as bone, cartilage, skin, and tendons.<sup>2</sup> It is therefore of great interest to understand the structure and function of collagen, in its many forms, in order to appreciate what role it plays in both healthy and unhealthy bodies. Since the advent of the electron microscope, this task has been made significantly easier, and much has been discovered about collagen.

Unfortunately, electron microscopy, although a very powerful tool, is not ideal for studying biological samples such as collagen. In order to be viewed by an electron microscope, samples must be made electrically conductive, meaning that collagen must be chemically fixed, dried, and then coated with a thin metallic film. This means that by the time of imaging, the samples are far removed from their original in vivo state and have a substantial amount of introduced artifacts. In fact, most microscopy methods are less than ideal because the biological sample must be dried, coated with a metal, or made to fluoresce by tagging with highly charged fluorescent

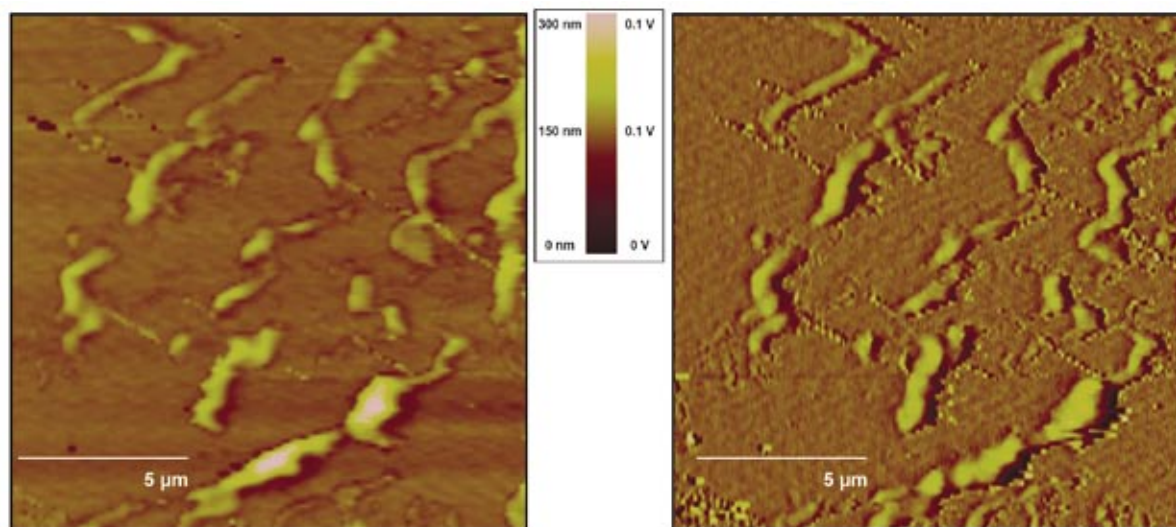
dyes. Atomic force microscopy (AFM) is an ideal technique to get realistic, high-resolution images of biological samples such as collagen, with conditions similar to in vivo conditions.

AFM provides topological information about a sample with nanometer-order resolution. This is accomplished by scanning a tip across a sample and using a laser to detect deflections in the tip position due to topological features.<sup>3</sup> In tapping-mode microscopy, the mode used in this study, the tip oscillates throughout the duration of imaging at its resonance frequency. Changes in the amplitude of this oscillation can be measured and correspond to the topography of the surface being imaged. This method is ideal for biological samples, such as collagen, for two reasons. First, tapping-mode does little damage to soft biological samples. The tip contacts the sample only very briefly, and with not very much force, and this usually prevents soft samples from being damaged.<sup>4,5</sup> The second reason is that AFM can be performed in liquid mode. This means that a biological sample can be imaged in liquid similar to the liquid present in vivo. This method of preparation both minimizes introduced artifacts and presents a more accurate picture of the biological sample.<sup>6</sup> In addition, imaging of live cells is also possible in a liquid environment. It is for these reasons that AFM was chosen as a method to image collagen fibers in an attempt to better understand their structure and function in conditions similar to in vivo conditions.

## **Background**

To date, more than 25 different types of collagen have been identified. The most abundant collagens are the fibril-forming

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**Figure 1:** AFM images of collagen type IV on AP-Mica. (Scan rate of 1.0 Hz.) Topography (left), amplitude (right).

collagens: types I, II, and III.<sup>2</sup> These collagen monomers contain three proteins arranged in a triple helix with nonhelical ends. The monomers are then arranged into pentamers (also known as microfibrils), and variable numbers of pentamers associate into the ultimate collagen fibrils. Cross-linking holds the fibrils together.<sup>7,8</sup>

Type I collagen was used in this study. This type of collagen constitutes the major component of the extracellular matrix of connective tissue. It is also found in skin, tendons, and bones and is the major type of collagen in the organic osteoid. It usually forms thicker bundles than types II and III and is considered most important in determining mechanical stability of bone.<sup>6,7</sup> Investigative studies, with both atomic force microscopy and other techniques, already have been done to determine type I collagen structure.

These have shown type I monomers to be only about 300 nm long by 1.5 nm in diameter, while the type I fibrils have diameters that range from 30 to 300 nm in diameter.<sup>8</sup> Some atomic force microscopy studies have been done on type I collagen in air and have shown larger oligomers of collagen with diameters from 400 nm to 600 nm.<sup>7</sup> Nonetheless, most of these studies have shown these fibrils to be cross-linked with others in different arrangements, depending on the particular test. Collagen is a substance that shows a great deal of polymorphism, and depending on the conditions, collagen molecules can form a wide variety of different structures.<sup>9</sup>

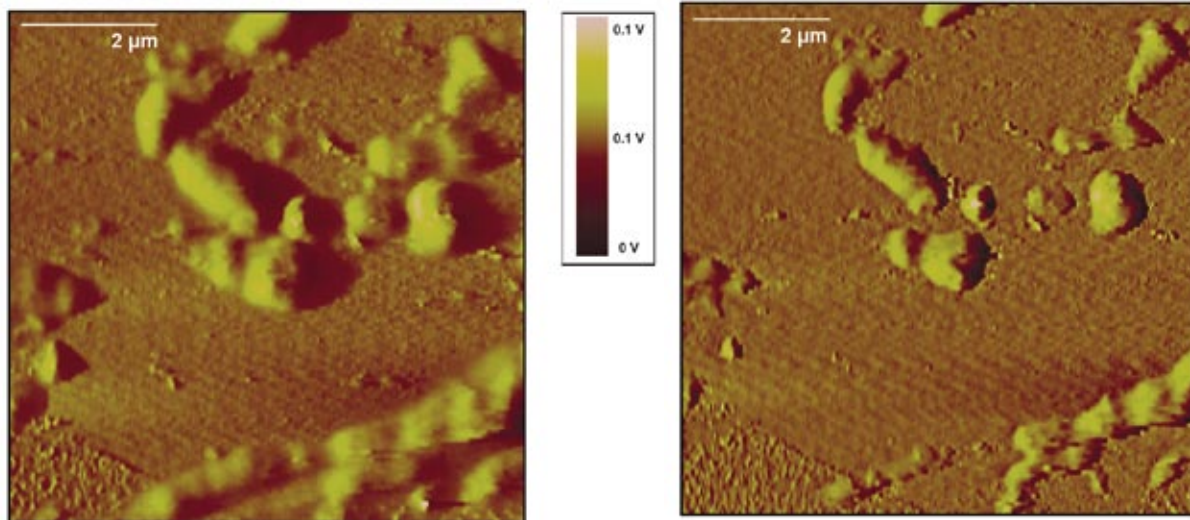
### Approach

In this study, the goal was to investigate nanometer-scale topographical structures of collagen fibers assembled in liquid

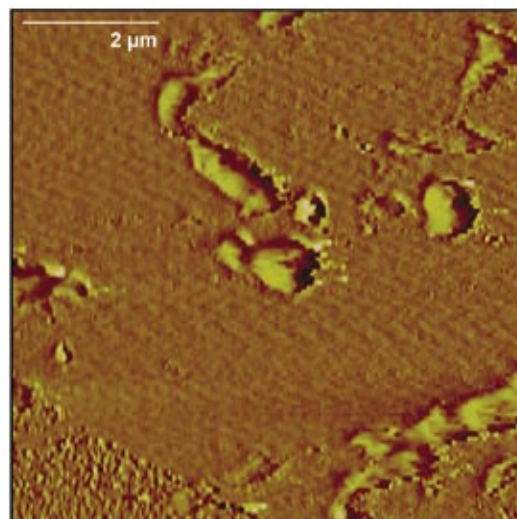
under conditions similar to tissues in vivo. Samples of collagen fibers were scanned with a Digital Instruments DI 3100 atomic force microscope and Veeco probes DNP-20S in order to gain a topographical image. This was done in the hope of establishing a starting point for the investigation of the structural properties of these fibers, which have not been decisively established to date.

### Sample Preparation

Three different types of collagen samples were prepared. The first, a sample of collagen IV, was used as a preliminary sample in order to determine the effectiveness of the AFM for these types of studies. (Type IV collagen, while similar in some ways to type I, is located only in the basal membrane of blood vessel walls. The type IV samples were used only to establish effectiveness of equipment and techniques, not to compare results with



**Figure 2: AFM images of oscillation amplitude for collagen type IV on AP-Mica. Amplitude set point values of 0.46 V, 0.41 V, 0.34 V (clockwise from left to right) were employed. (Scan rate of 1.0 Hz.)**



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those of type I.) The type IV samples consisted of collagen fibers in phosphate-buffered saline (PBS) solution, incubated for 2 hrs at 37°C. A drop of this solution was then placed on freshly cleaved AP-Mica.<sup>5</sup> (AP-mica is mica that has been modified to make the surface positively charged to facilitate binding with negatively charged collagen fibers.) This freshly cleaved AP-mica was then allowed to sit for 40 min in order for the fibers to fix to the surface. The sample was then washed with deionized water, and PBS buffer was added to facilitate liquid-AFM study.<sup>5</sup>

The second set of samples prepared consisted of collagen I fibers affixed to glass slides. In order to facilitate binding of the collagen to the glass, the slides were first coated with 1 mg/ml poly-L-lysine, and then a 5 µg/ml solution of the collagen in 0.01M HCl was added (pH

2). The samples were then neutralized with ammonia hydrochloride vapor for 2 min, and the solution was replaced with PBS (pH 7.4). The samples were then incubated for 30 mins at 37°C, and the PBS was aspirated, leaving a thin layer of PBS salt. Deionized water was added to these samples to reconstitute the PBS buffer solution immediately before scanning.

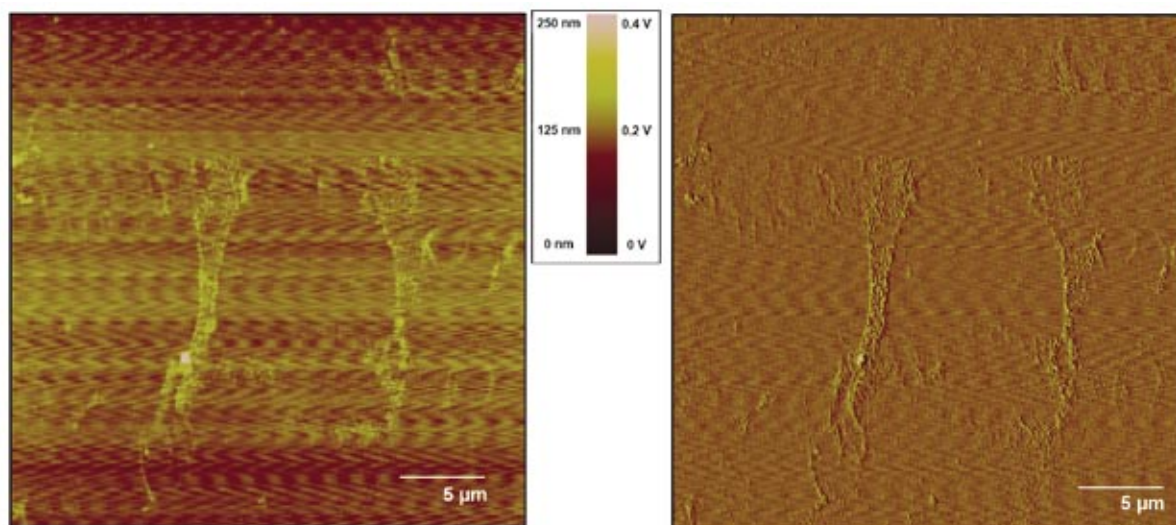
The third set of samples was prepared in exactly the same way as the second, but instead of the glass slides being coated with poly-L-lysine, they were cleaned with ethanol before adding the collagen.

### *AFM Imaging*

As stated previously, atomic force microscopy was performed in liquid, with tapping-mode microscopy. A glass cantilever holder, designed for liquid studies, was used, as well as oxide-sharpened silicon nitride cantilever tips (Veeco probes, DNP-20S). These tips

have a radius of 10 nm and are ideal for obtaining high-resolution images even when imaging soft samples. Just before imaging, a drop of solution (either PBS or DI water) was placed on top of the AFM cantilever, and another drop was placed on the substrate being scanned. The cantilever was then made to approach the surface until the two separate drops of liquid combined into one. This assured that the area being scanned was completely immersed in liquid.

Tapping-mode microscopy is based on measuring changes in the amplitude of oscillation of the silicon nitride cantilever. A piezo tube in the AFM is used to oscillate the cantilever at its resonance frequency in liquid, usually between 8 and 9 kHz. The tip of the cantilever is then made to contact the surface and proceeds to scan the surface. In our studies, the scan rate was between



**Figure 3:** AFM images of collagen type I on poly-L-lysine-coated glass slide. (Scan rate of 1.001 Hz.) Topography (left), amplitude (right)

1.0 and 2.0 Hz, depending on what gave best image quality. If the tip encounters areas of increased height as it scans across the sample, the amplitude of oscillation of the cantilever will decrease initially. The AFM, however, is programmed with an amplitude set point that must be maintained. In order to do this, the AFM feedback control will lower the sample slightly, increasing the oscillation amplitude to its initial value. The change in the sample height over the course of scanning can then be correlated to a topographical map, showing the height information of the sample. An additional map is generated based on the initial changes in amplitude at the beginning of topographical features. This means that when the tip first contacts a heightened area, the initial decrease in oscillation amplitude can be measured and correlated to a map corresponding to oscillation amplitude. The end result,

then, is two topographical maps: The amplitude map is generally more precise at the edges of features, while the height map is more accurate elsewhere.

### Results and Discussion

#### *Collagen IV on AP-Mica*

After scanning the first set of collagen IV samples, we found that AP-mica constituted an appropriate substrate for the binding of collagen fibers. When tests were performed with untreated mica, no collagen images were obtained, implying the samples did not fix to the substrate. As shown in Figure 1, however, there clearly are structures bound to the AP-mica substrate. In this image there seem to be two types of structures, one linear and one globular; since scanning of collagen IV was not pursued further, it is hard to know exactly what these structures represent. However, proof that

the observed features correspond to soft matter is given in Figure 2. The three images in Figure 2 are all images of the same feature but with different oscillation set points. As the oscillation set point decreases, the force that the cantilever applies to the substrate increases, meaning that the first image represents the lightest tapping. We can see that as the force of tapping increases, the size of the feature decreases, implying that this is a soft material.<sup>10</sup> In addition, we see that the location of the features changes only slightly, due to piezoelectric drift,<sup>11</sup> demonstrating again that these features are indeed bound to the substrate.

#### *Collagen I on Poly-L-Lysine-Coated Glass Slide*

In the second set of experiments, collagen IV was replaced with the more general type of collagen, collagen I. Due to a number of experimental difficulties, only

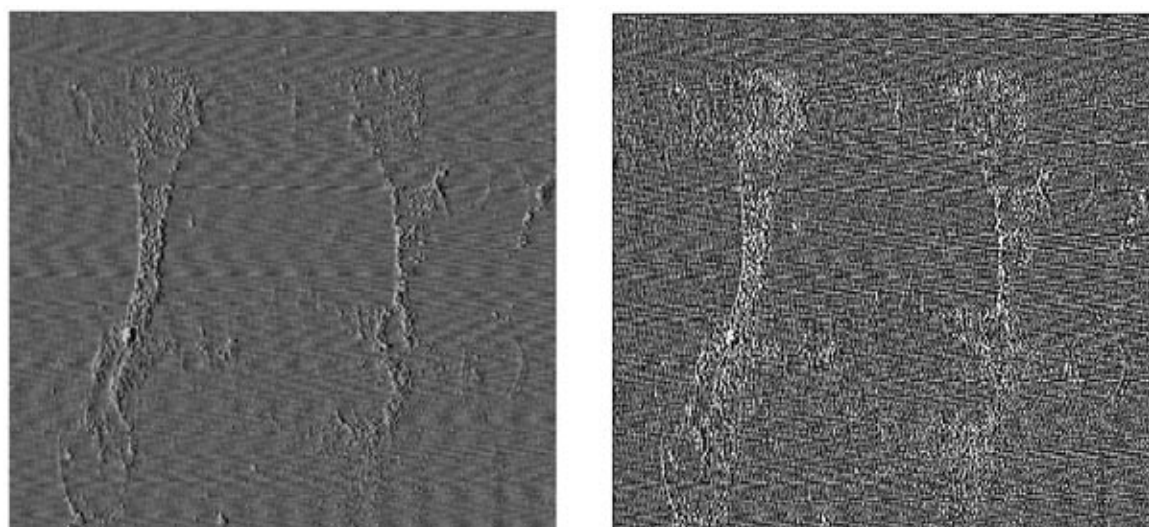
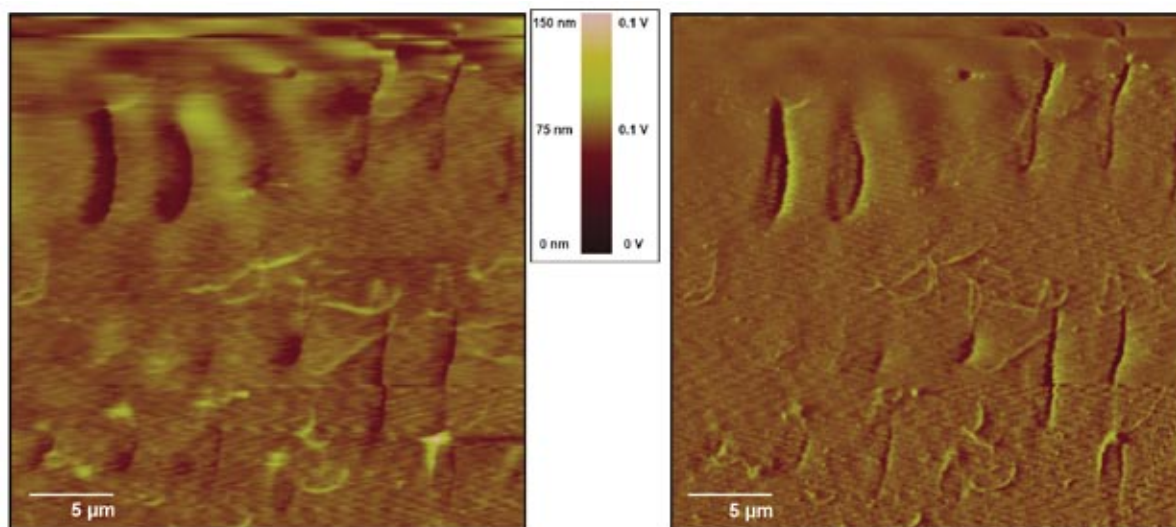


Figure 4: Digitally processed images of Figure 3 (right) using the MetaMorph Image Acquisition and Image Processing Program (Universal Imaging, Inc.).

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**Figure 5:** AFM images of collagen type I on ethanol-cleaned glass slide. (Scan rate of 1.001 Hz.) Topography (left), amplitude (right).

one image was obtained for this sample, but it is very informative nonetheless. Figure 3 shows the original obtained images, while Figure 4 shows these images after being digitally processed using the MetaMorph Image Acquisition and Image Processing Program (Universal Imaging, Inc.).

We can see from these images that there are structures clearly present on the surface of the glass. These structures are about 16  $\mu\text{m}$  long and range between 1 and 5  $\mu\text{m}$  wide. This is too large to be a single collagen fibril, but the images suggest that this is a conglomeration of collagen fibrils, rather than just one. The second image in Figure 4 shows a series of small dots, possibly representing individual collagen fibrils. These structures appear to be around 60 to 200 nm, which would be reasonable for a fibril; unfortunately, the image resolution is only

117 nm, and a higher-resolution image is necessary to get a true sense of the size of these individual fibrils. However, this structure certainly suggests a network of cross-linked collagen fibers, as are present in vivo.

In addition, these images could provide valuable information about self-assembly of collagen networks. The two features visible in these images are nearly identical in their overall structure. This suggests something about their method of assembly. If these structures indeed represent a network of cross-linked collagen fibers, it is very interesting and informative that two separate networks developed in much the same way. Again, further studies would need to be done to determine how these fibers assemble, but this image provides a valuable starting point for this investigation.

### *Collagen I on Ethanol-Cleaned Glass Slide*

The results obtained from the ethanol-cleaned glass slide were drastically different from the results from the poly-L-lysine-coated glass slide. Figure 5 shows many scattered collagen fibers, seen in the middle of the images. The larger dark lines that appear in pairs do not represent collagen, as it can be seen from the height map that they represent depressions in the surface instead of protrusions. However, the smaller scattered light-colored structures probably represent collagen fibers, as they are approximately 2.4  $\mu\text{m}$  long, 99.6 nm tall, and 176 nm wide. This size could correspond to a single collagen fibril, but it cannot be said for sure because the resolution in this image is only 117 nm.

After processing the images again with MetaMorph (Figure 6), we can see that these structures clearly overlap but do not

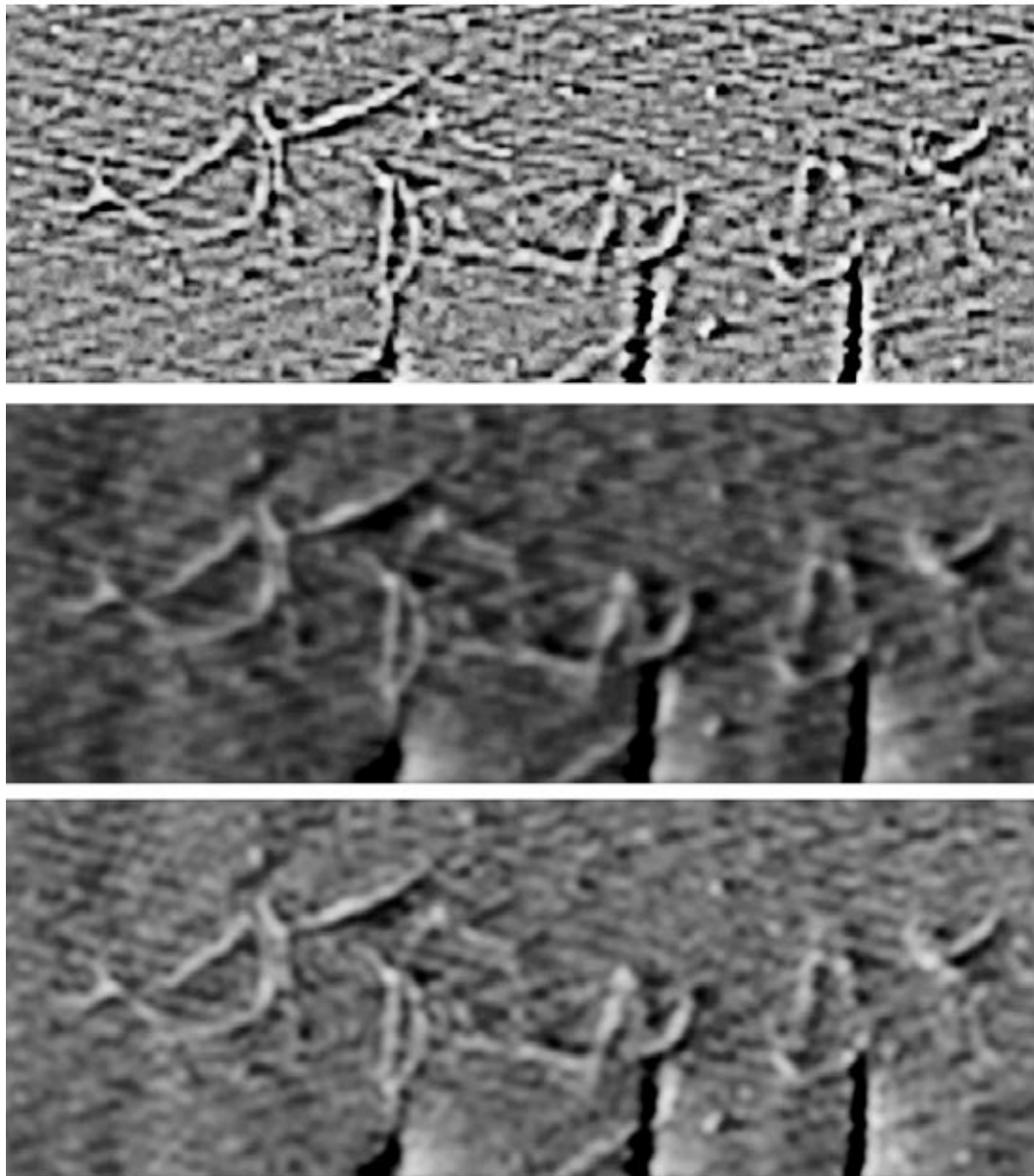
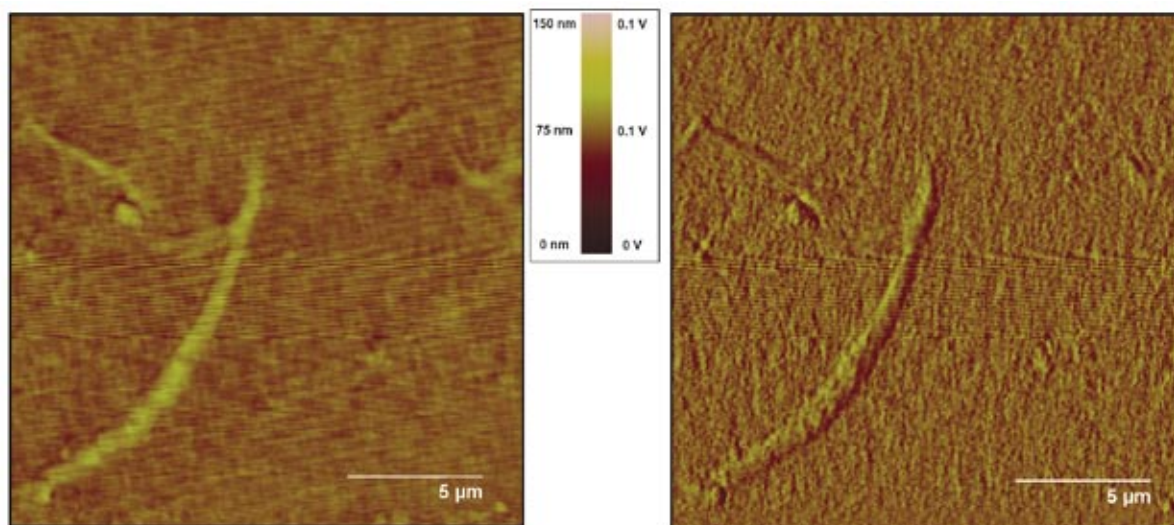


Figure 6: Digitally processed images of Figure 5 (left) using the MetaMorph Image Acquisition and Image Processing Program (Universal Imaging, Inc.).

## Imaging of in Vitro Collagen Fibers by Atomic Force Microscopy (*continued*)



**Figure 7:** AFM images of collagen type I fibril on ethanol-cleaned glass slide. (Scan rate of 2.001 Hz.) Topography (left), amplitude (right).

seem to show the repetitive cross-linking pattern characteristic of collagen, and seen on the slide with poly-L-lysine.

In order to gain more information about these potential fibers, higher-resolution images must be obtained. Figure 7 shows a closer view of a collagen fiber, with a lateral resolution of 29.2 nm. The fiber is 5.87 μm long, 379 nm wide at its widest point, and 92.6 nm tall, meaning this could be a collagen fibril. However, we can see from Figure 8 that although the dimensions of the fibril seem probable, the actual surface texture seems to be an artifact. After image processing and removal of background patterns, we see that the fiber and the background still have the same surface texture, implying that this texture is not representative of the fiber itself. Most likely, this texture is due to PBS residue that was not reconstituted upon addition of deionized

water. In future studies, PBS would be added in liquid form right before imaging to prevent deposition of PBS salt onto the substrate.

Perhaps the most interesting image is shown in Figure 9. In this figure, and in the processed image (Figure 10), the ends of several collagen fibers are visible and appear to be composed of several smaller structures wound around each other into the larger collagen fiber. This suggests that these structures are indeed collagen fibrils, and these smaller strands represent microfibrils wound together. We can see that several of these fibrils have these unwound ends, indicating that they have potentially been broken. This theory is supported by the structure on the upper-right corner of the original image, which appears to have been broken and consequently shows this striated structure. This seems

to suggest that, unlike the poly-L-lysine samples, something in the preparation of the ethanol-cleaned samples caused the collagen fibers to be broken and prevent them from cross-linking sufficiently. This would be an interesting relationship to examine further if more conditions were controlled in order to determine exactly what is causing this behavior.

### Conclusions

Atomic force microscopy (AFM) is a relatively easy method of gaining information about collagen. In this study we found that AP-mica, glass treated with poly-L-lysine, and glass treated with ethanol all bound to collagen and provided a smooth surface to scan with the AFM. These three substances all cause different structures of collagen to form.



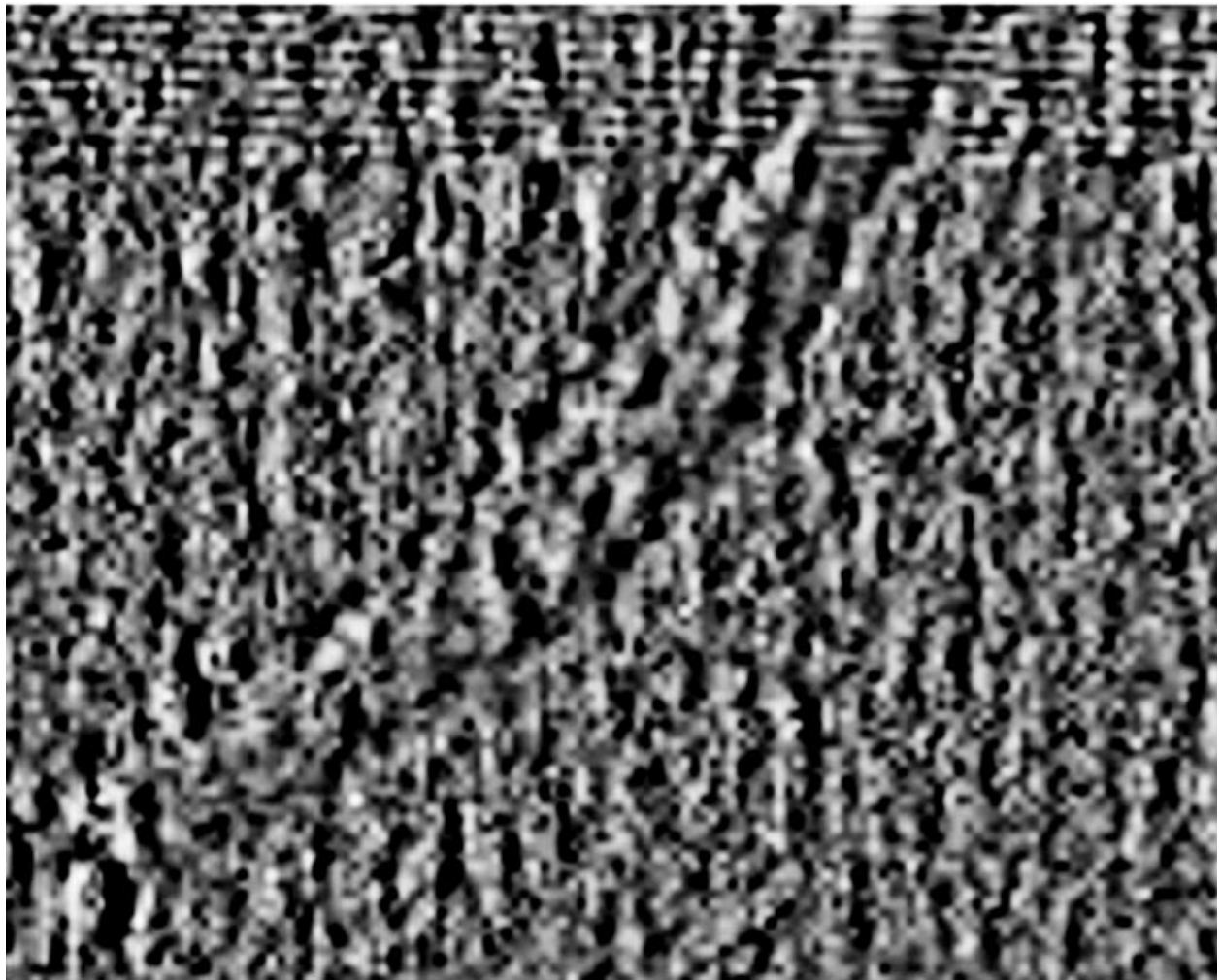


Figure 8: Digitally processed images of Figure 7 (left) using the MetaMorph Image Acquisition and Image Processing Program (Universal Imaging, Inc.).

Imaging of in Vitro Collagen Fibers by Atomic Force Microscopy (*continued*)

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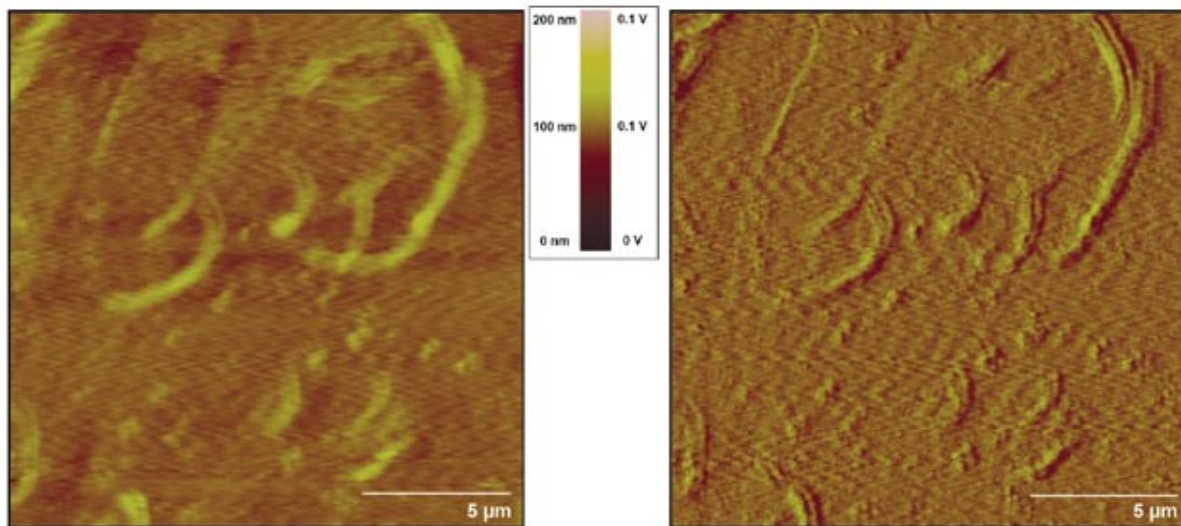


Figure 9: AFM images of collagen type I fibrils on ethanol-cleaned glass slide. (Scan rate of 1.001 Hz.) Topography (left), amplitude (right).

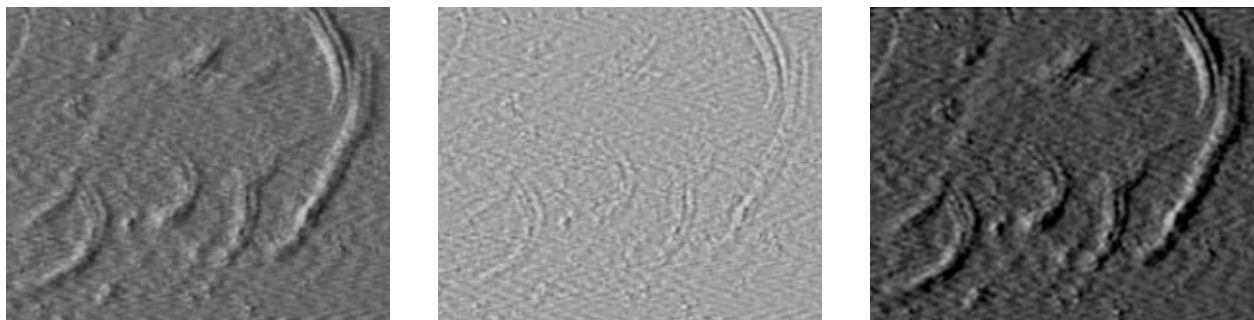


Figure 10: Digitally processed images of Figure 9(left) using the MetaMorph Image Acquisition and Image Processing Program (Universal Imaging, Inc.).

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This study represents a starting point in using AFM to examine collagen fibers in conditions similar to those in vivo. Ultimately, AFM could play a vital role in understanding the structural properties of collagen type I fibers, as well as how these properties change in the many different conditions present in the human body.

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