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Quantitative evaluation of the collagen organization in dermis by AI-assisted AFM microscopy

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1. Introduction

Aging induces significant disorganization of the skin tissue, particularly in the dermis where fibrous proteins such as collagen or elastin form a dense network that gives the skin its mechanical properties. Assessing the effects of aging on the hierarchical structure of collagen is a challenge, as the effects can be induced from the molecular level to the microfibril bundles. Histological observation is the most common *in vitro* technique to assess collagen quality, however only the bundle scale can be visualized. To obtain higher resolution information, down to microfibrils, electron microscopy and biophysical techniques are required. Atomic force microscopy (AFM) provides high-resolution images of the surface of skin cross-sections, showing the collagen microfibrils arranged parallel to each other in dense bundles, and the famous structure in bands spaced 65 nm apart. While the images are spectacular, their quantitative analysis does not go beyond a simple visual inspection [1]. A Fast Fourier Transform analysis has proved interesting, but it is limited only to the orientation of the fibres [2]. To quantitatively characterize the quality of microfibrils organization visualized by AFM, we have developed a new image analysis processing based on artificial intelligence (AI).

2. Materials and Methods

2.1. Samples. The ability of our AI-based analysis of AFM images to detect and quantify aging effect on collagen structure was achieved by comparing one untreated piece of an explant to two pieces of the same explant that were chemically "aged" by carbamylation, either by sodium cyanate or urea. The explants were prepared under usual *ex vivo* conditions (maintained in a survival medium at 37°C in a humid atmosphere, enriched with 5% CO2).

Carbamylation treatments were repeated 8 times for 13 days. The explants were then cryofixed and stored at -80°C.

2.2. AFM observations. About 20 µm thick transverse cuts of the three explants were mounted on a microscope slide and allowed to air-dry for at least 24 hours before the measurements. They were examined using a Multimode 8 Bruker microscope associated with a NanoScope V controller (Bruker) with a silicon-etched cantilever with a full tip cone angle of ~40° and a tip radius of curvature ~10 nm. Four high resolution images (3x3 µm^2) were collected for each explant type at 500 µm from the top of the epidermis at a scan rate of 1.0Hz at 512x512 pixels resolution.

The AFM images of dermis clearly show the striated structure of collagen microfibrils and thus make it possible to characterize the cohesion between the neighbouring microfibrils, *i.e*. an inter-fibrillar characteristic supposed to be linked to the firmness of the tissue [3]. In the untreated sample, the collagen microfibrils appear parallel and tightly packed, whereas the collagen packing is looser and less regularly oriented for the two treated samples, which is expected after aging (Figure 1).

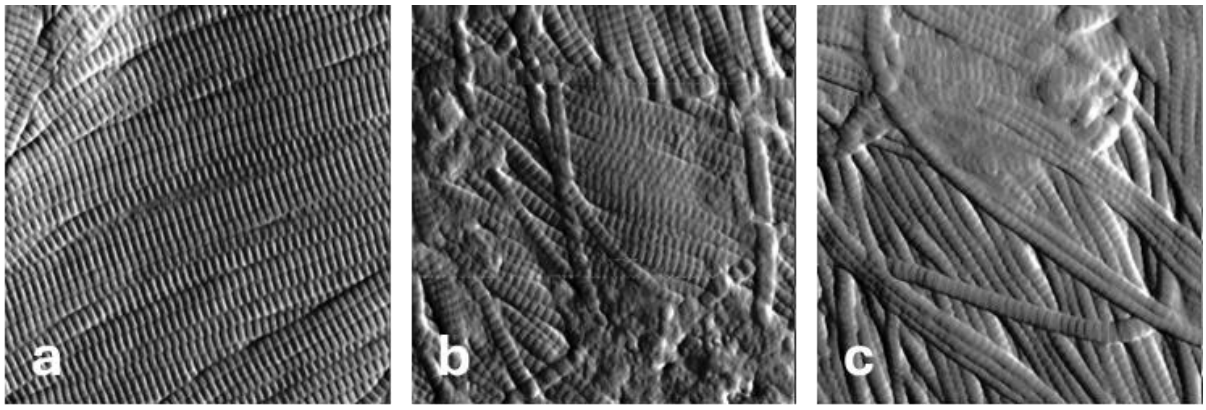

Figure 1. Typical AFM images of the three explants (field of view: 3x3 µm^2)
(a) untreated (b) treatment with sodium cyanate (c) treatment with urea

2.3. AI treatment. To go beyond simple visual inspection and try to quantify the observations, we have developed an AI-based processing. The AFM images (3*3 µm²) of the collagen acquired in dermis were gridded into 4x4 sub-images. A neural network was trained on 24 sub-images (0.75*0.75 µm²) to recognize and score three types of collagen structures: mark 1: disorganized microfibrils; mark 2: criss-crossed microfibrils; mark 3: rectilinear and parallel microfibrils (Figure 2) using a Novitom software. The rate of success of the prediction test (95%) was deemed high enough to validate the training. Then, for each AFM image, prediction was performed on all sub-images (0.75*0.75 µm²) that received a mark.

With this analysis protocol, the quality of the collagen organization at high resolution can be evaluated either by the average sore calculated from all the AFM images acquired on each type of explants or by the distribution of each organization type (mark 1, 2 or 3) depending on the treatment condition. This allows a numerical comparison of the level of interfibrillar organization of collagen microfibrils for the three explant conditions.

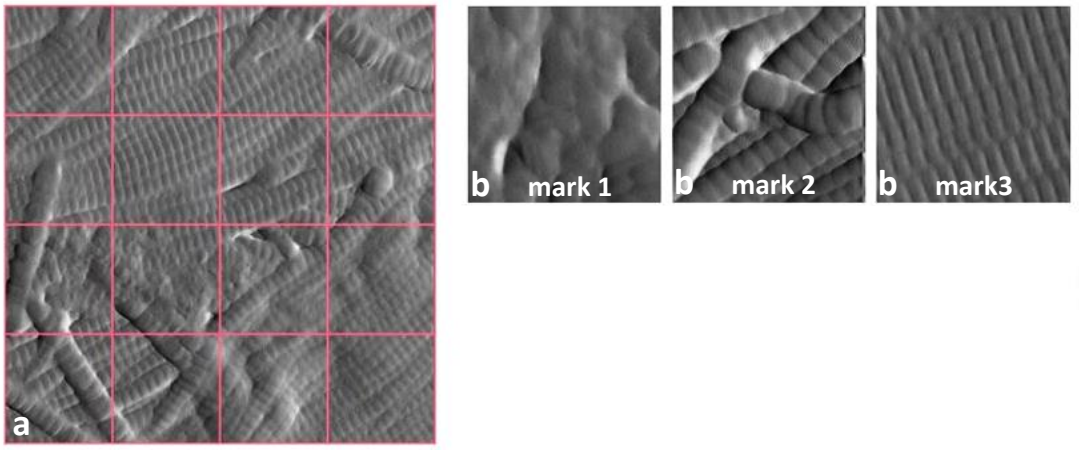

Figure 2. (a) example of 3x3 µm^2 AFM image showing the 16 sub-images
(b) examples of three areas with low, medium and good interfibrillar organization used for the AI training with respectively marks 1, 2 and 3

3. Results

The histogram describing the results (Figure 3) clearly shows an effect of carbamylation. Mark 3 is observed for 94% of subzones, wich indicates good cohesion between collagen microfibrils, while marks 1, 2 and 3 are more distributed for carbamylated samples. The mean AI-based score for the untreated explant (2.91) is significantly higher than that of the two carbamylated samples (resp. 2.11, 2.14), thus confirming and quantifying the carbamylation-induced alteration and validating the analysis protocol.

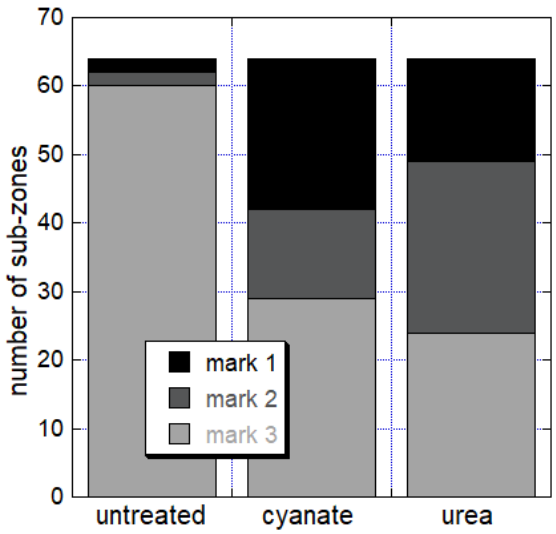

Figure 3. Histograms showing for each type of explant the number of sub-images out of the 64 in total with a given mark 1, 2 or 3

4. Conclusion

This original approach provides objective structural information on collagen organizations in the dermis, at inter-fibrillar structural levels. It offers information complementary to histological observation, biometric measurement or Omics studies. It is a sensitive approach that can detect minimal changes and provides striking images of the collagen network. This approach represents a promising new test to corroborate the effect of anti-aging active ingredients. Interestingly, its use is not limited to the simple case of anti-aging products, it can indeed benefit all treatments supposed to change the organization of collagen, such as anti-stretch marks or healing treatments.

References

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