Atomic force microscopy characterization of corneocytes: effect of moisturizer on their topology, rigidity, and friction

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Background/purpose: Atomic force microscopy (AFM) is a novel technique for skin characterization.

Objectives: To develop AFM tests for characterization of the outermost epidermis layer, corneocytes. As an example, the effect of moisturizer on the corneocyte properties is studied.

Methods and materials: Topology, rigidity, and friction (between individual corneocytes and AFM probe) of the top layer of corneocytes were measured by means of Veeco DM3100 AFM. Quench moisturizing cream was applied daily on the forearm of five volunteers for a period of 9 days. The skin flakes were collected before and after the treatment using Cuderm tape strips. No additional treatment of flakes was performed before the measurements.

Results: A protocol for the AFM study of corneocytes is developed. After the treatment, we observed overall smoothening of the corneocyte surface, an increase of friction, and a decrease of rigidity (the Young modulus).

Conclusion: AFM can be used as a very sensitive tool for early detection of changes in corneocytes.

Key words: skin flakes – corneocyte – atomic force microscopy – Young’s modulus – topology – friction

In recent years, research related to skin has incorporated many novel techniques like confocal scanning laser microscopy (1–3), confocal Raman microscopy (4), electron microscopy (5, 6) ultrasound microscopy (7, 8), electro-impedance techniques (9), etc. However, atomic force microscopy (AFM) has not been used extensively to study the skin. AFM is based on the detection of forces acting between a sharp probe, the AFM tip, and a sample surface (10–12). The tip is attached to a very flexible cantilever. Any motion of the cantilever is detected and recorded by means of a computer. While scanning over the surface of interest, the AFM system records the deflection of the cantilever with sub-nanometer precision. The AFM technique is more than just simply microscopy. One can think about the AFM tip as a microscopic ‘finger’ with a nanosize apex. Following this analogy, one can use this microscopic finger to touch the surface to detect its rigidity (rigidity force microscopy, nanoindenter), surface stickiness (chemical force microscopy), measure friction (friction microscopy), etc.

As for the skin research, so far, only the volumetric analysis of single corneocytes has essentially been performed by means of AFM (13). In principle, similar measurements could be performed using other techniques, for instance, optical surface profilometry (14–16). At the same time, the AFM provides researchers with truly unique information. For example, it can be used to measure surface roughness with a resolution far surpassing optical and electron microscopy. In addition to being a microscope, it is also a force-measuring apparatus. It allows measurement of the elasticity (rigidity) of the surfaces [e.g. of cells and molecules (17–20)], friction between the AFM probe, and the surface of study (21–23).

In the present work, we demonstrate the use of AFM to characterize the physical properties of corneocytes, one of the most abundantly found investigative material on the outermost epidermis layer. Specifically, we measure individual corneocytes to extract the following properties: surface corrugation on a submicron level, rigidity (the Young modulus), and friction coefficient between a silicon nitride AFM probe and corneocyte surface.

Apart from fundamental interest in learning the above properties, the use of AFM may be of
considerable practical interest. The sensitivity of the AFM method is so high that it allows detecting very early changes. This, for example, may reduce the time required for clinical studies. Here, we analyze the change of the measured parameters of corneocytes after treatment with a moisturizer. We developed a protocol to detect the changes in the collected corneocytes with AFM after 9 days of treatment with the moisturizer (Olay Quench™ body cream). The protocol was tested on a group of five individuals. After the treatment, we observed overall smoothening of the corneocyte surface, an increase of friction, and a decrease of rigidity (the Young modulus). This may represent a substantial time reduction when carrying out clinical studies.

Method and Materials

Materials
Cuderm Dark (carbon filled) adhesive tape strips (D-square skin indicator D200, CuDerm Corp., Dallas, TX, USA) were used for skin corneocyte collection. This tape was advantageous both for force measurements (allowing ease of dissipation of electrical charges that may create additional long-range forces, suppressing the AFM signal) and for optical prescreening; see the latter. A commercially available Quench™ moisturizer (Procter & Gamble Corp., Cincinnati, OH, USA) was used in the study.

Subjects
Five healthy Caucasian males 25–56 years old participated in this study after giving informed consent. None of them had any visual abnormalities on the test areas, which could affect the AFM measurements. The volunteers were not checked for any allergies as it was difficult to know at that moment. The flakes were collected from the inner part of the forearm.

Treatment with moisturizer
The cream was applied daily to the inner part of the forearm by each subject in the morning. The treatment was carried out for 9 days. The skin flakes were collected before the treatment and also on the 10th day, after about 24 h from the last application of the cream. This was important to separate the therapeutical effect of moisturizer from the immediate physical effect of softening and lubrication of corneocytes.

Sample preparation: collection of flakes
The corneocyte skin flakes were collected using Cuderm Dark (carbon filled) adhesive tape strips before and after the treatment with moisturizer. The collected flakes were first screened with the help of an optical microscope. This allowed avoiding regions with a high aspect ratio of flakes (flakes not properly attached to the adhesive tape) and flakes with internal air gaps; see the description below for more details.

After collecting and optical prescreening mentioned above, the flakes tended to dry. This may result in the alteration of all flake parameters. We observed that after some period of time (~24 h), these parameters stabilize. However, long-term stability was not studied. Analyzing the short-term stability, we monitored the change in the measured parameters (topology, rigidity, and friction). All three parameters remained almost unchanged within 1 h under ambient conditions (room temperature was ~20°C, and humidity ~50–70%). After 3 h the rigidity increased between two and four times, topology changed within 10%, while no change was found in friction. Thus, we performed our measurements within the first hour of collecting flakes. It should be noted that low humidity (~20%), observed in countries with cold climate during the winter time, could prevent effective use of the method because of very fast drying of the corneocyte samples collected. Therefore, humidity control is highly recommended during these experiments.

Optical prescreening of corneocytes for AFM imaging
Air gaps beneath the corneocyte surface, loosely attached corneocytes, or too high outstanding flakes may result in various AFM artifacts in measuring the geometry, rigidity, and friction. To exclude such artifacts, the flakes under study were prescreened with the AFM built-in optical microscope system with a side-illumination using a fiber optic light (Fig. 1).

In addition to the dissipation of charges mentioned before, the black carbon tape strips allowed visual observation of the above-mentioned issues as well as the regions filled with air gaps. Figure 2a shows air gaps seen as rainbow features in the video microscope with side illumination. Figure 2b demonstrates the same features shown in a higher contrast when using dark-field mode optical imaging (done on a stand-alone optical microscope).
AFM data collection: general description
A Nanoscope™ Dimension 3100 (Digital Instruments/Veeco Inc., Santa Barbara, CA, USA) AFM was used in the present study. A standard cantilever holder cell for operation in air was used. Standard integrated pyramidal silicon nitride AFM cantilevers/tips (Digital Instruments/Veeco Inc.) were utilized. To obtain topographic information on corneocytes, AFM images were collected in the contact mode of operation.

To collect sufficient statistics when measuring rigidity, the force-volume mode of operation was utilized. The force-volume mode provides information about both the surface topography and the force curves simultaneously. This is important because the models to quantify the measurements have been developed for a sphere over the surface of known geometry, mostly a plane. Thus, we processed force curves only over relatively flat areas of the flake (the slope $<10–15\degree$). The force curves were collected over the areas of ca. 5 $\mu$m$^2$. The AFM probe moves up and down during the force collection with a frequency of 2 Hz. The global position of the AFM probe was controlled by the built-in video system, which allows observation of areas from 150 x 110 to 675 x 510 $\mu$m$^2$ with a 1.5 $\mu$m resolution.

The friction was found from the friction loops (21–23) using at least three different load forces. The lateral spring constant of the cantilevers was not calibrated in force units. The vertical spring constant was found using the built-in Nanoscope software option (which is based on the resonance method).

AFM data collection: protocol

1. The AFM tip is positioned near the appropriate flake/corneocyte by means of optical microscopy (built-in video microscope). The corneocyte for imaging should be relatively flat and air-gap free (area that does not show any interference or ‘rainbow’ pattern as described above).

2. A quick scanning is performed on an area of 20 x 20 $\mu$m$^2$ with a scanning speed of 3–4 Hz and an initial imaging resolution of 128 x 128 pixels. This allows to check whether the scanning area is suitable for further data collection (if the height variations are within the measurable range, there is no saturation).

   a. If the area is sufficiently flat (no saturation in the image), the same area is scanned with a speed of 3–4 Hz and a resolution of 512 x 512 pixels for further topology analysis.

   b. If it is hard to find a sufficiently flat area, the scan area can be decreased to 10 x 10 $\mu$m$^2$ (or 15 x 15 $\mu$m$^2$), and the imaging is performed on this area with a speed of 3–4 Hz and a resolution of 512 x 512 pixels for further topology analysis.

Fig. 1. DM3100 atomic force microscopy with a side-illumination fiber optic light (shown by an arrow), which was used for the optical prescreening.

Fig. 2. (a) Air gaps are seen as rainbow features when using the AFM DM3100 built-in video microscope and the side illumination shown in Fig. 1. Image (b) shows the same but taken in the dark-field mode using a stand-alone optical microscope (shown for better presentation of the air-gap feature).
3. A relatively flat area of $5 \times 5 \mu m^2$ is then chosen by zooming out of the area imaged in the previous step. This area is then used for the force-volume analysis. This analysis is processed with a collection speed of 9 Hz (vertical ramping). The relative trigger deflection (stops approaching the surface when the cantilever deflection exceeds the value of the trigger) is set to 100 nm for a regular silicone nitrite-integrated cantilever with a spring constant of $\sim 0.1 \text{N/m}$.

4. A smaller area of $\sim 1 \times 1 \mu m^2$ is then used for the collection of friction data. The friction loops were first monitored while the AFM tip scans along the surface. The most representative friction loop was then chosen (the slow scan parameter is disabled at this time to remain on the same scan/friction line). The friction signal was then collected for three different load forces (corresponding to three different setpoints, e.g. 1, 2, and 4 V). It is recommended to return back to the minimum load (1 V in the above example) to check whether the friction signal reverts to what was observed initially. If this does not happen, the measurements should be repeated with a decreased maximum load force. This maximum force is to be used in the measurements on the other corneocytes.

5. To find the coefficient of friction (COF), the absolute value of the load forces has to be measured. This is done by switching to the force calibration mode. The force curves are collected for each setpoint that was used for collection of the friction data (for the above example, 1, 2, and 4 V).

$\text{Definition of the measured parameters}$

The following parameters were used to characterize corneocytes:

1. The Surface Fiber Density Index (SFDI) is used to characterize the smoothness of the surface (24). It is defined as follows:

$$\text{SFDI} = \frac{\text{surface area} - \text{projected area}}{\text{projected area}} \times 100\%$$

where ‘surface area’ is the topographic area of the chosen part of the image, while the ‘projected area’ is the area of the same chosen part but projected on a flat substrate (in the other terms, the underlined area).

2. The Young modulus can be determined using the JKR model (25). The JKR analysis assumes a linear stress–strain relation, and so may be successfully applied when the forces are small (low stress) and deformations are modest (small strain). In addition, it assumes that there are no long-range interactions, but takes into account possibly large adhesion. The Young modulus $E$ in this model is defined by the following formula:

$$E = \frac{3}{4} \frac{1}{R^{1/2}} \frac{dP_{\text{JKR}}}{d(h^{3/2})}$$

where $P_{\text{JKR}} = \frac{1}{\sqrt{3}} P_{\text{off}} P_1^{5/2} = \frac{4}{3} R^{1/2} E' h^{3/2}$, $P_{\text{off}} = kd$;

$$P_1 = (3P_2 - 1) \left[ \frac{1}{2} (P_2 + 1) \right]^{1/3}, P_2 = \left[ Z_{\text{def}} + 1 \right]^{1/2}.$$

Here, $Z_{\text{def}}$ is the deflection of the cantilever, $d$ is the cantilever deflection at the point where the tip loses contact with the surface (the point of maximum negative deflection), $v$ is the Poisson ratio, $R$ is the curvature radius of the tip, $h$ is the penetration of the tip when deforming the sample, and $k$ is the spring constant of the cantilever. $P_{\text{off}}$ is called the pull-off force and $P_{\text{JKR}}$ is the reduced applied force or JKR applied force.

3. COF is defined as COF = $F/N$, where $F$ is the friction force (between the AFM tip and the corneocyte surface) and $N$ is the load force normal to the surface.

$\text{Data processing}$

In order to obtain the SFDI and coefficient of friction from the topography (deflection) images, Nanoscope software v. 5.12r4 (Veeco Inc.) was used. MicroMechanical Analysis software v. 1 (by Vladimir Tsukruk’s Laboratory, Georgia Tech, Atlanta, GA, USA) was used to process the force volume data files, and consequently, to determine the Young modulus. Significant difference of averages was defined for $P$ level of 0.05.

$\text{Results}$

The AFM data yielded the statistical difference between the treated and the untreated skin flake samples collected from five subjects who participated in the study (all Caucasian males 21–56 years old). The corneocytes were collected and processed immediately as described. The data were collected on four corneocytes for each subject to check its variability. A representative AFM
image of a corneocyte is shown in Fig. 3. The SFDI is calculated for each $5 \times 5 \mu m^2$ part of the image using a built-in option in the AFM software. This brings up to 16 values of SFDI for a $20 \times 20 \mu m^2$ image.

The Young modulus was calculated using force data averaged over $\sim 200$ individual force curves. An example of an averaged force curve is shown in Fig. 4a. Using the JKR model as described above, the Young modulus can be calculated for different AFM probe penetrations. This is shown in Fig. 4b. The fact that the modulus is scattered around the same value (does not depend on the penetration) proves the validity of the model. If the opposite is observed, the surface is not a homogenous medium. In general, it is normal to expect no homogeneity of corneocytes. However, here, we measure the deformation of a rather thin region, the penetration of $\sim 10$ nm. We expect homogeneity in such a thin layer of material.

Figure 5a shows a graphical representation of the frictional signal ($F$), which is calculated as a difference between the friction signals collected for the probe moving in opposite directions (trace and retrace). Both curves are recorded using a 512 pixel resolution. Thus, $F$ can be found for each of 512 pixels. However, the friction signal arising from tilted surfaces can contain both friction and geometrical resistance (additional force needed for the AFM probe to climb a bump). To avoid the confusion of friction with this additional force, signal $F$ was collected over a relatively flat area of the corneocyte. Typically, signal $F$ is constant over such areas.

To determine COF, one needs to normalize friction $F$ on the normal force $N$ as described in the Methods section. Figure 5b shows the cantilever deflection plot as obtained from the AFM software. The corresponding normal force $N$ is found as the spring constant of the cantilever $k$ multiplied by $D$, the difference between the
assigned zero deflection $d = 0$ nm (corresponding to the setpoint), and the actual zero deflection when the probe is far from the surface (flat parts of the deflection curves).

The data processed as described are collected in Table 1 for the corneocytes taken before and after the treatment with moisturizer. Each column also shows the ratio of the average values after and before the treatment. The ages of the subjects are given for reference. The values averaged for all subjects are shown in Fig. 6 as histograms. One standard deviation corresponds to the variability between the subjects.

## Conclusion/Discussion

A protocol for the AFM study of human corneocytes is developed. Following this protocol, a significant difference was observed between the topology, rigidity, and coefficients of friction on corneocytes collected from the topmost layer of human skin (inner part of the forearm) before and after treatment of the moisturizer. While topology showed an overall change towards the increase of smoothness (the decrease of the SFDI index), it was significantly different only for three subjects and insignificant for two others. The coefficient of friction showed an overall increase of friction. However, the increase was significant for three subjects and insignificant for two others (one subject was the same as the one with the insignificant change of the SFDI index). The Young modulus changed quite consistently. For all subjects, the average rigidity declined quite noticeably, although variability (one standard deviation) was quite high in the definition of the Young modulus.

The following conclusions can be made. Overall, AFM showed a difference in the flake properties before and after treatment with the moisturizer. While different subjects may react

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**TABLE 1. Atomic force microscopic data**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Subject 2: 56 years</th>
<th>Subject 3: 30 years</th>
<th>Subject 4: 21 years</th>
<th>Subject 5: 22 years</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surface Fiber Density Index (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>6.0 ± 1.4</td>
<td>7.0 ± 0.4</td>
<td>4.7 ± 1.0</td>
<td>9.5 ± 1.1</td>
</tr>
<tr>
<td>After</td>
<td>6.1 ± 0.8</td>
<td>5.6 ± 0.7</td>
<td>4.8 ± 0.5</td>
<td>2.6 ± 1.1</td>
</tr>
<tr>
<td>Ratio</td>
<td>~ 1</td>
<td>0.8</td>
<td>~ 1</td>
<td>0.27</td>
</tr>
<tr>
<td>Coefficient of friction</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.51 ± 0.08</td>
<td>0.49 ± 0.15</td>
<td>0.54 ± 0.18</td>
<td>0.54 ± 0.21</td>
</tr>
<tr>
<td>After</td>
<td>1.3 ± 0.34</td>
<td>0.42 ± 0.10</td>
<td>0.67 ± 0.16</td>
<td>1.3 ± 0.74</td>
</tr>
<tr>
<td>Ratio</td>
<td>2.55</td>
<td>0.9</td>
<td>1.2</td>
<td>2.41</td>
</tr>
<tr>
<td>The Young modulus (MPa)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>24 ± 4</td>
<td>66 ± 22</td>
<td>60 ± 26</td>
<td>70 ± 17</td>
</tr>
<tr>
<td>After</td>
<td>19 ± 5</td>
<td>49 ± 16</td>
<td>37 ± 11</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>Ratio</td>
<td>0.79</td>
<td>0.74</td>
<td>0.62</td>
<td>0.31</td>
</tr>
</tbody>
</table>

The average values and one standard deviation are shown.
differently to the moisturizer, apparent trends are clearly seen after the averaging. Corneocytes become smoother, develop more friction, and become softer after the treatment. It should be noted that the observed variability is an intrinsic characteristic of corneocytes rather than an error of measurements. Therefore, we can still use the average as an indicator of the treatment. Comparison of the averages for different subjects shows a significant difference for all three measured parameters on corneocytes collected before and after a 9-day treatment with moisturizer. Thus, the AFM technique can be used as a fast screening device for the development of new skin care products.

Acknowledgements

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References


Fig. 6. Atomic force microscopic data averaged on all human subjects. The top portion of each bar shows one standard deviation. COF, coefficient of friction; SFDI, Surface Fiber Density Index.

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