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# Age determination of blood spots in forensic medicine by force spectroscopy

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## Abstract

We present a new tool for the estimation of the age of bloodstains, which could probably be used during forensic casework. For this, we used atomic force microscopy (AFM) for high-resolution imaging of erythrocytes in a blood sample and the detection of elasticity changes on a nanometer scale. For the analytic procedure we applied a fresh blood spot on a glass slide and started the AFM detection after drying of the blood drop. In a first step, an overview image was generated showing the presence of several red blood cells, which could easily be detected due to their typical ‘‘doughnut-like’’ appearance. The consecutively morphological investigations in a timeframe of 4 weeks could not show any alterations. Secondly, AFM was used to test the elasticity by recording force–distance curves. The measurements were performed immediately after drying, 1.5 h, 30 h and 31 days. The conditions were kept constant at room temperature (20 °C) and a humidity of 30%. The obtained elasticity parameters were plotted against a timeline and repeated several times. The elasticity pattern showed a decrease over time, which are most probably influenced by the alteration of the blood spot during the drying and coagulation process. The preliminary data demonstrates the capacity of this method to use it for development of calibration curves, which can be used for estimation of bloodstain ages during forensic investigations.

*Keywords:* AFM; Force spectroscopy; Forensic medicine; Age determination; Blood spots

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## 1. Introduction

Blood analysis including blood cell measurement is one of the most basic items of hematological testing which is indispensable in health examination, disease diagnosis and treatment. The common lab test is used to diagnose and monitor the body's response to diseases. Some tests measure the components of blood itself and others examine substances found in the blood to identify abnormal function of various organs. In forensic sciences the examination of bloodstains represents a major application during crime scene investigation. There exists a lot of reliable methods for the detection and identification of blood spots. For the evaluation of suspected bloodstains solutions such as phenolphthalein, tetramethylbenzidine can be used, as they change color when they come into contact with peroxidase or hemoglobin in the blood [1]. For the detection of even minute amounts of blood traces the presumptive luminol chemiluminescence test is widely used in forensic practical work [2]. It is further possible to unambiguously attribute the blood to a certain individual by using molecular biological techniques, such as genetic fingerprinting [3].

However, the determination of the age of a blood spot remains an unsolved problem in forensic routine work. For more than hundred years forensic scientists are engaged in finding a methodology, which allows determining the exact age of a dried bloodstain. Since then several approaches were

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proposed to have potentially solved this important problem during crime scene investigation, but none of these could ever be established in forensic practice. An attempt was undertaken by evaluating the time differences of the solubility of bloodstains of different ages [4]. In control experiments it turned out, that both methods considerably diverge from the real age of an investigated bloodspot. More recently, new methods were tested such as remission spectrophotometry [5] or electron-spin-resonance measurements [6], which can detect an age-dependent increase of signal intensity of methhemaglobin, non-hem-iron molecules and organic radicals. Concordantly, these methods proved to have a high error rate, which only allow a rough estimation of the age of a bloodstain and does not excuse the high technical expense. In more recent times chromatographic methods were tested for the use in dating blood spots. Inoue et al. [7] used high pressure liquid chromatography (HPLC) to measure the quantitative compound of the globin chains of the red blood dye hemoglobin. They found a decrease of the α-chains related to the heme, the color defining prosthetic group of the red blood dye hemoglobin, with increasing age of a blood spot. The measurements of the standardized bloodstains revealed high deviations and revealed not to be practicable for forensic routine work. Moreover, a mobile application of this method for the use directly at a crime scene investigation seems not to be realizable. Taken together, all described methods proved to be not applicable in forensic routine work, as the results of the age measurement of the bloodstains provided too high deviations compared to the real age. Most of these attempts rely on advanced technical equipment and, therefore, does not allow the application directly at a site of crime. Additionally, a part o the sometime rare trace material has to be consumed for the use of these approaches, so that it will be lost for further important analysis, like molecular genetic investigations.

We present a new methodology which could be used for the age determination of dried bloodspots during crime scene investigation. Therefore, an AFM was used as a nanoindentor to monitor age-related changes of the elasticity of bloodstains under standardized conditions.

Since the invention of the AFM by Binnig et al. [8], indentation experiments are generally applied in measuring elastical properties. Nanoscaled materials are probed with the AFM due to it's high lateral and vertical resolution down to 0.01 nm. In contrast to other hardness tester the force resolution of the AFM can reach ranges of $10^{-4}$ nN [9]. The application of AFM–force spectroscopy is widespread and used for measuring polymer systems [10,11], bone elasticity [12], collagen [13] or cells [14–16].

To investigate the alteration of the elasticity of blood samples with the AFM by force spectroscopy, force–distance curves were applied to the blood spot. To compare two samples it is sufficient to calculate the slopes in the upper linear part of the force–distance curves (see Fig. 1). For calculation of the Young's modulus a model has to be fitted to the measured data. A detailed calculation procedure can be found in the next section.

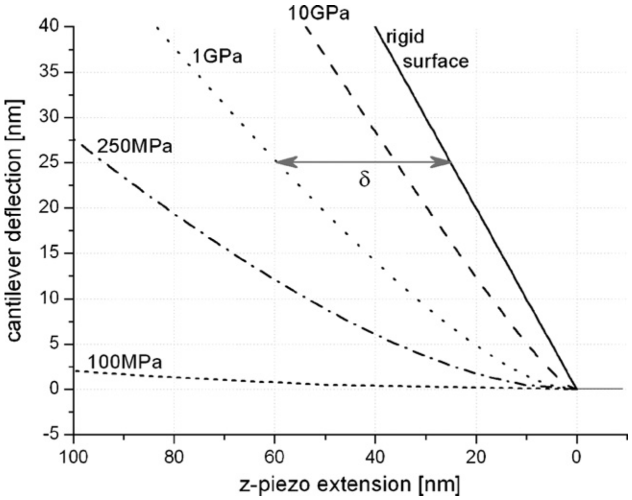

Fig. 1. Force–distance curves of various elastic samples calculated with the Hertzian theory. At rigid surfaces the AFM tip cannot penetrate into the sample, therefore the cantilever deflection corresponds to the extension of the $z$-piezo. The softer the sample the more penetrates the tip into the sample, therefore the slope is very smooth. To get the elasticity modulus (Young's modulus, $E$) the Hertzian or similar models have to be fitted to the measurement curves.

## 2. Materials and methods

### 2.1. Preparation of the blood samples

For the AFM investigations a few drops of capillary blood from a healthy human volunteer was drawn from the fingertip by using blood lancets. The blood drops were applied to a glass slide and dried at room temperature.

### 2.2. AFM microscopy

For morphological characterization of the blood samples an AFM Topometrix Accurex (Atos, Germany) was used. It was operated in contact mode under ambient conditions, with 35% relative humidity, using NSC12F (noncontact silicon cantilever) cantilevers (Mikromasch, Estonia). The spring constants of the non-contact probes (NSC) was 0.3 N/m ($F$). The nominal resonant frequency of the NSC cantilever was 21 kHz ($F$). The nominal tip radius was specified <10.0 nm. Image analysis was performed using SPIP software (Image Metrology, Denmark).

### 2.3. Force spectroscopy and data analysis

Force spectroscopy was carried out with an AFM of Molecular Imaging/Agilent using Si cantilever (special made NSC15 high force constant, pyramidal tip shape, cone half angle $\alpha = 15^\circ$, cantilever typical cantilever thickness 4.8–5.5 μm, resonant frequency nominal: 405 kHz, spring constant nominal $k_c = 80$ N/m, Mikromasch, Estonia). For determination of the effective spring constant the Sader method was applied [17]. This procedure can be easy applied using the length, the width and the resonant frequency in fluid, typically air, with the accompanying quality factor $Q$. In this case the spring constant $k_c$ is independent from the thickness and the density. The exact measurement of both parameters is non-trivial and additionally defects in the material are taken into account because of the usage of the resonant frequency. All comparative experiments were carried out with the same cantilever in ambient conditions with a spring constant of $k_c = 74$ N/m. The detailed calculation procedure for calculating the Young's modulus is presented in the following.

The indentation of an AFM tip into soft or hard samples can be modeled using the Hertzian contact mechanics [18]. Indentations of an infinitely hard body into a hard elastic half space (small indentations with the parabolic part of the tip) with a normal force $F$ leads with this theory to [19,20]:

$$F_{\text{paraboloid}} = \frac{4}{3}\frac{E}{1-\nu^2}\delta^{3/2}\sqrt{R} \tag{1}$$

where $\delta$ is the indentation depth, $E$ the Young's modulus, $\nu$ the Poisson ratio and $r$ is the tip radius. For incompressable materials the Poisson ratio is at 0.5. The applied force can be calculated with the deflection of the cantilever which is considered as a tightened spring by Hook's Law:

$$F = k_c d \tag{2}$$

where $k_c$ is the spring constant and $d$ is the deflection of the cantilever.

The deflection of the cantilever depends on the indentation of the tip into the elastic half space or rather the sample. The $z$-piezo extends and the distance of the extension $z$ is splitted into the deflection and the indentation depth ($\delta$) by:

$$z = d + \delta \tag{3}$$

With these Eqs. (1)–(3) the Young's modulus can be expressed for hard samples as followed:

$$E_{\text{paraboloid}} = \frac{3k_c(d-d_0)(1-\nu^2)}{4\sqrt{R}[(z-z_0)-(d-d_0)]^{3/2}} \tag{4}$$

where $d_0$ and $z_0$ are the corresponding values of the cantilever deflection and the $z$-piezo extension at the contact point. Eq. (4) can now be written for soft samples in the contact regime as:

$$z = \left[\frac{3k_c(d-d_0)(1-\nu^2)}{4\sqrt{R}}\right]^{2/3} + (d-d_0) + z_0 \tag{5}$$

To calculate the Young's modulus the model (Eq. (5)) has to be fitted to every force–distance curve. For the evaluation the average of approach and retract curve was taken. The spring constant ($k_c = 74$ N/m) was determined by the Sader method [17], the radius of curvature ($R$, $R \approx 200$ nm), determining the tip shape was determined using an scanning electron microscopy (SEM) image.

In Fig. 1 typical force–distance curves calculated with the Hertzian theory are presented. The horizontal line on the right side shows the behaviour when the tip is far away from the sample. The tip approaches to the sample and a force between tip and sample arises. It could be either attractive or repulsive. In case of attractive forces a snap in of the tip to the sample surface occurs while with repulsive forces the cantilever is bended up. Both events are not the case with our blood samples.

The force–displacement curves were processed using the software Microcal Origin and Microsoft Excel, to evaluate the slopes and the Young's moduli. For calculation of the Young's modulus Eq. (5) was applied to the measured data as described before.

## 3. Results

### 3.1. AFM imaging of the bloodstains

The AFM imaging of the samples revealed no morphological differences between the bloodstains following drying and after 31 days (see Fig. 2). The first row displays a series of images recorded on the freshly dried specimen. In the subsequent rows the same region of the bloodstain is visualized after 1, 2 and 4 weeks. In the overview images some small cracks in the bloodstain can be observed, which are already present in the first images taken immediately after drying of the blood. In the detailed pictures the erythrocytes can be easily identified due to their typical doughnut-like shape. Most interestingly, neither the erythrocytes nor the cracks in the bloodstain showed any morphological alterations during the observation period of 31 days.

### 3.2. Elasticity measurements on the bloodstains

On three different aged bloodstains (immediately after drying, 30 h and 31 days), force spectroscopy was carried out with the AFM, using the tip as a nanoindentor. Force–distance curves were recorded on different areas of the blood spot. In Fig. 3A an AFM image with a typical area for recording force–distance curves on the blood spot is displayed. The measurements of the elasticity on the blood were carried out on the thick center area to exclude effects of the hard glass substrate. Fig. 3B shows a typical force–distance curve of the 31 days old sample.

Fig. 4 displays the calculated Young's modulus of the force–distance curves with the individual standard variations of the samples. The average values of the samples differ significantly from each other. This clearly shows, that the elasticity of the bloodstain decreased with the age of the sample.

However, with regard to the standard variation the Young's moduli are very similar and the error ranges show a slightly overlap mainly in the lower values. Fig. 5 shows the huge variation of the individual slopes of the force–distance curves. The values are highly dependent on the measuring point.

## 4. Discussion

The unique elastic properties of the red cell membrane and their ability to withstand deformation stresses during circulation have been characterized by biomechanical investigations as well as biophysical studies. While the biochemistry of the system including various protein and lipid components is well established, the structural basis of the components is still under debate [21]. First insight into the red cell network structure and its viscoelastic properties derived from investigations using micropipette aspiration [22]. In more recent studies the AFM played a major role in characterizing the membrane assembly and its nanomechanical properties. The unfolding of spectrin repeats, a major component of the red cell cytoskeleton, by using the AFM provided deep insight into the mechanical stability and the structure of this protein [23]. In medical research, the AFM was mainly used to investigate morphological alterations of red blood cells, which show a relation to a certain disease. Kamruzzahan et al. [24] observed the surface topography of red blood cells of patients with systemic lupus erythematosus and compared it to those of healthy donors. Thereby, they were able to detect characteristic circular shaped holes at the surface of the red blood cells from the patients under physiological conditions, which could correlate with described functional deficiencies in red blood cells [25]. In an earlier study Zachee et al. [26] visualized uremic red blood cells with the atomic force microscope. The ultra-morphological images confirmed the existence of uremic echinocytes and demonstrated the capability of the AFM to study cell surfaces.

The imaging of altered erythrocytes due to mechanical stress represents another application of the AFM in blood research. Under various physiological conditions artificial organs occasionally traumatize the blood cells, which can lead to the critical liberation of hemoglobin molecules of erythrocytes. Ohta et al. [27] were able to show that the fine structure of the

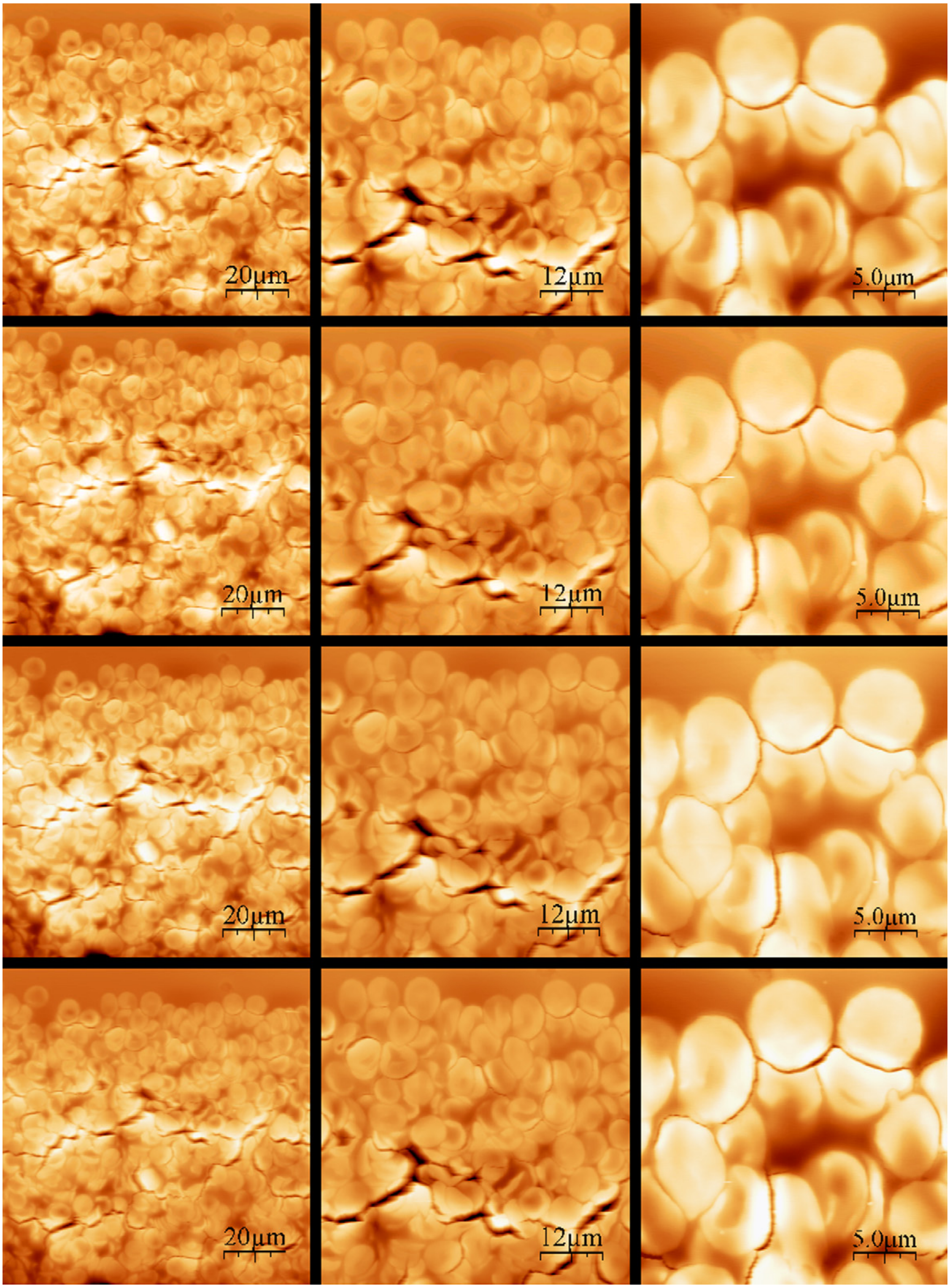

Fig. 2. Results of the morphological investigations of a dried blood spot by AFM. The illustration is divided into rows and columns. The AFM images in the rows correspond to the individual measuring days. The columns display identical scan areas depending on the alteration time of the blood spot. The first series (first row) was scanned after 1.5 h, the second series after 1 week, the third series after 2 weeks and the fourth series after 4 weeks. The morphology independent on the scan range (100, 60, 25 μm) does not change with the alteration time.

red blood surface was changed drastically by artificially induced shear stress. Moreover, they found that the surface roughness increased with the exposure time and could be correlated to the liberated hemoglobin concentration. In another study the influence of different agents including phospholipids, low ionic strength media and drugs on the shape of human erythrocytes were investigated and characterized by the AFM [28]. The artificially produced abnormally shaped

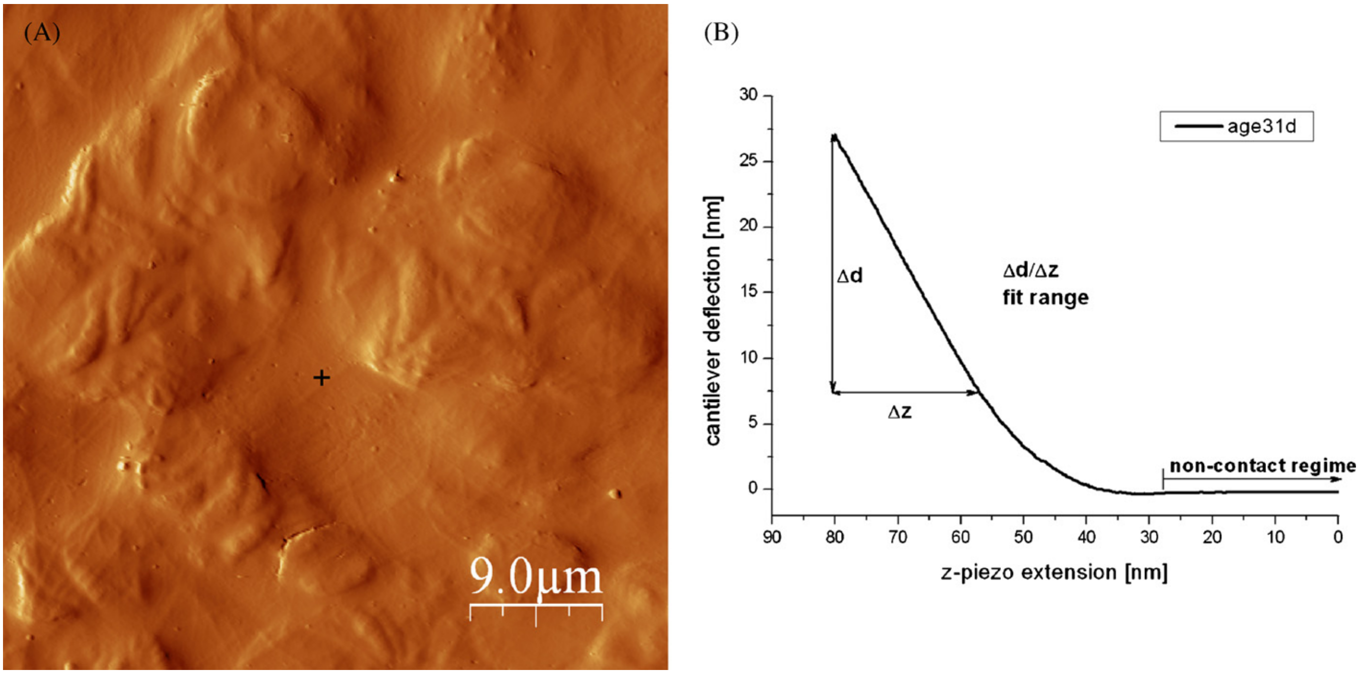

Fig. 3. Typical force–distance curve with corresponding AFM image showing the measuring spot. (A) AFM image of a typical area for recording force–distance curves on the blood spot. The measurements of the elasticity on the blood were carried out on the thick center area to exclude any effects of the hard glass substrate. The measuring points were distributed randomly over the sample. As an example, one of these measuring spots is marked on the AFM image (cross). (B) Typical force–distance curve of the 31 days old sample. The horizontal line on the right side of the curve is referred to as zero line, where tip and sample are not in contact. To exclude the influence of surface effects with unknown magnitude the fit is performed in the upper part of the curve marked as $\Delta z$ and $\Delta d$. During the fitting procedure the Young's modulus $E$ and the $z$-value of contact point $z_0$ are the calculated fitting parameter of Eq. (5).

erythrocytes were further compared with cells that occur with high incidence in blood pathologies, such as spherocytosis and anisopoichilocytosis. In all these medical related studies the AFM was used to image erythrocytes and detect differences in shape or cell wall surfaces. In only a few investigations the AFM was used to study elastic properties of cells by recording force curves and relate the differences of normal and altered cells to pathological conditions. Force spectroscopy of normal and cancerous human bladder cells revealed a Young's modulus of about one order of magnitude higher of normal cells compared to the cancer cells [29]. The differences in elasticity could probably be attributed to changes in the cytoskeleton due to oncogenic transformation. In a further study Lekka et al. [29] probed the erythrocyte stiffness of red blood cells from patients suffering from coronary disease, hypertension and diabetes mellitus and compared it to erythrocytes derived from healthy donors. Analysis of the recorded force curves provided an

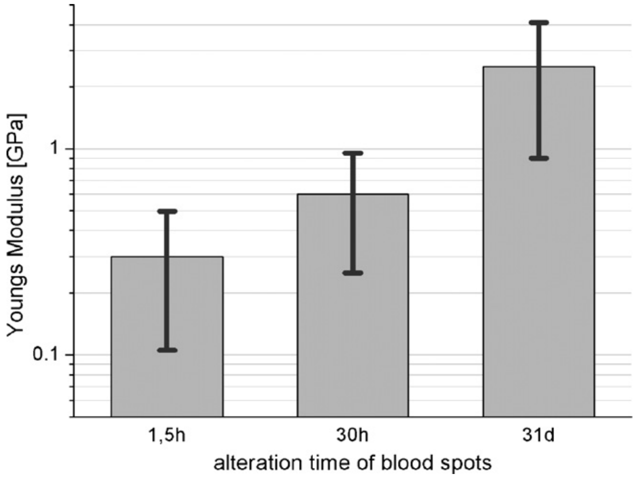

Fig. 4. Elasticity change vs. alteration time. The evaluation of the Young's moduli indicates an elasticity change depending on the alteration time. The bloodstain was measured after 1.5 h, after 30 h and after 31 days. Every column was calculated from more than 100 individual $F$–$d$ curves. The whisker displays the standard deviation of the force curves.

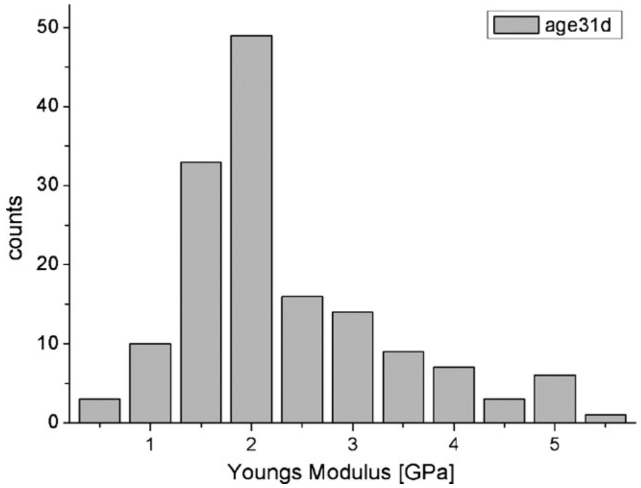

Fig. 5. $F$–$d$ curve histogram of measurement day 31. The variation can be attributed to the inhomogeneous assembly of the blood spot. The $F$–$d$ curves are recorded on the surface, influences from the bulk below cannot be excluded.

increase of the stiffness in samples from cigarette smokers and diabetes mellitus patients. This observation confirmed other biochemical studies, which have shown that chemical changes in the erythrocyte membrane, especially the ratio between phospholipids and cholesterol content can lead to an increase in cell wall rigidity in patients suffering from diabetes mellitus [30].

Until recently, the AFM was only introduced in a few specialized applications in forensic medicine, such as the examination of line crossings in documents [31]. In a recent study a first attempt was undertaken to detect time-dependent changes of the erythrocyte morphology [32]. The authors found cellular changes of erythrocytes after exposure in air for several days by using dynamic mode atomic force microscopy. They observed cell shrinking and fissures after 0.5 days and at 2.5 days of exposure nanometer scaled protuberances occurred which increased in number with time. Based on these results, Chen and Cai proposed that the alteration of the red blood cells could be used to estimate the time of death. However, the erythrocytes were not investigated under conditions to be found at a crime scene, but were mixed with an anticoagulant (EDTA), immunolabelled, deposited on mica and fixed manually. Therefore, it cannot be ruled out that the pre-treatment of the blood influences or enhances the observed cell wall alterations.

In contrast to this work, we investigated a bloodstain on a glass slide without any kind of pre-treatment or chemical modifications. Thereby, we could not detect any morphological alterations of the red blood cells over a time period of 4 weeks. The AFM images recorded during the whole observation period of the bloodstain showed no major differences (see Fig. 2). However, the recorded force–distance curves of our blood sample showed a clear increase of the stiffness with increasing age of the bloodstain. This represents the first application of force spectroscopy to investigate age related changes of dried blood, which could serve as a helpful tool in forensic science and serve as a new approach to solve a major problem during forensic casework, the age determination of dried bloodstains.

Nevertheless, we observed a few limitations during our investigations, which have to be clarified by further experiments. The measurements showed a high standard deviation, which can probably be explained by the non-homogeneous composition of the blood clot. During coagulation a fibrin network is generated in which platelets, erythrocytes, leukocytes and other blood components are embedded. Despite the fact, that the blood clot appears to be a homogeneous system, the single components seem to influence the elasticity parameters of the whole system in different ways. Moreover, there exists evidence that erythrocytes show some alteration in membrane elasticity and viscosity during in vivo ageing [33]. The age of the erythrocyte (1–120 days) could therefore have an influence on the measurements of the bloodspot. However, these differences are most probably negligible compared to the major elasticity changes during drying of the bloodstain. This holds particular true with respect to the fact, that we did not perform elasticity measurements on single blood cells, but considered the bloodstain as a complete system (see Fig. 3). The stiffness values of single erythrocytes and the influence of the measurement spot will be the major topic of our further studies, in which we are planning to measure the single components of the blood and its elasticity parameters by force spectroscopy. Together with these upcoming data we hope to be able to establish a tool, which can be used for the age determination of dried blood spots in forensic routine applications.

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