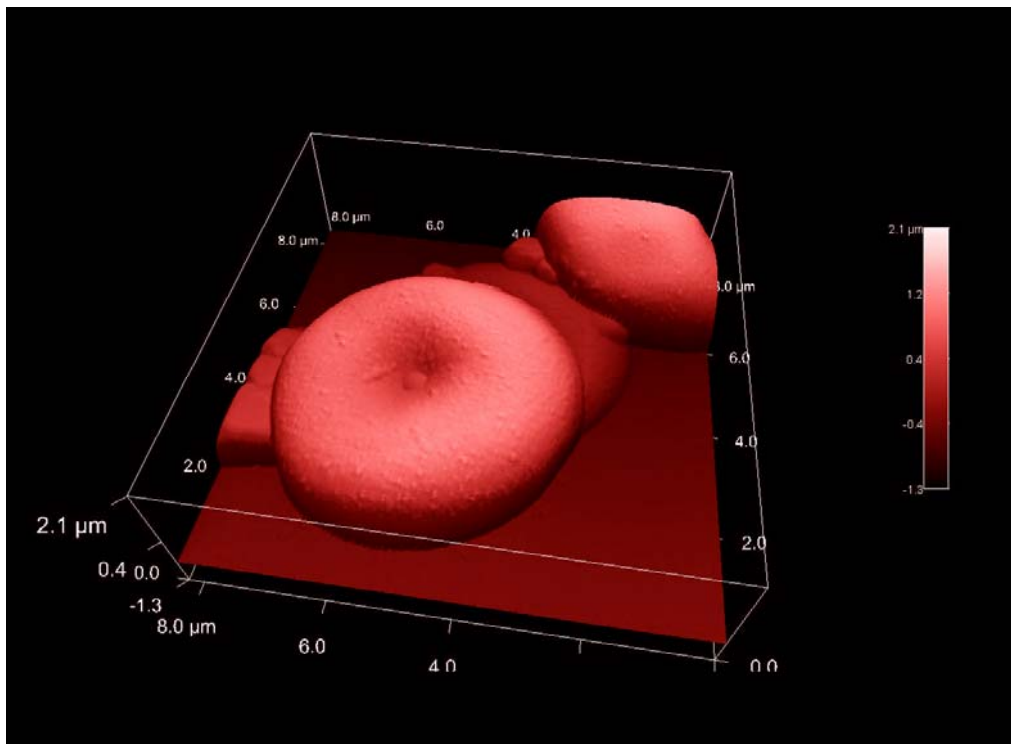


AFM Force mapping on healthy and EPO red blood cells.

Project work
“Interactions with surfaces”
134.114



by

Oliver Hekele
Matr.Nr.0026349

Christoph Gösselsberger
Matr.Nr.0425384

supervised by

Ao.Univ.Prof.Dr. Friedrich Aumayr
Univ.Ass.Dr. Ille C. Gebeshuber

Contents

1. Abstract	2
2. Introduction.....	3
3. Overview of Erythrocytes and EPO.....	5
4. The Atomic Force Microscope (AFM)	8
4.1. Contact mode	9
4.2. Dynamic mode	10
4.3. Force curves	11
5. Sample preparation	13
6. Force Mapping using MFP-3D and Igor Pro	14
6.1. Getting started	14
6.2. Save/Open Waves	15
6.3. Force Mapping	16
6.4. Analysis.....	18
6.5. Save Analysis Data	20
7. Measurement.....	22
8. Results.....	24
9. Discussion and Outlook	31
10. Acknowledgements.....	32
11. References.....	33
12 Examples of blood sample measurements	35

1. Abstract

In this project work we investigated if there is a difference in the structure and stiffness of red blood cells which are produced by body own erythropoietin or by synthetic erythropoietin (EPO). For this procedure we used the atomic force microscope (AFM), which offers the possibility to investigate the surface and stiffness of the red blood cell in more detail. For our experiments we pressed the tip of the cantilever with a certain force into the cell surface and investigated if there is a difference in the penetration depth of the tip. With the limited amount of samples investigated, we found no significant differences in the stiffness of the blood cells, but some abnormalities in one of our blood sample, which should be investigated in more detail. As a result of the AKH (Allgemeines Krankenhaus Wien / Vienna general hospital) doctors' interest in our work and technical possibilities with the AFM for medical research we consider to strengthen this collaboration on similar projects in the future.

2. Introduction

With the atomic force microscope (AFM) it is possible to investigate the soft surface of erythrocytes. The AFM provides an insight into the surface, structure and topography of the human red blood cells as well as the investigation of tip induced deformation of the cells. By using the force mapping mode of the AFM software one can mark certain points on the cell where the tip has to move to. With a certain force the cantilever tip can be pressed into the marked points of the cells.

Inspired by the abuse of erythropoietin (EPO) in serious sports (doping) we investigated if there is a difference in the structure and stiffness of red blood cells which are produced due to synthetic EPO and body own EPO.

For our experiments we compared the stiffness of blood samples of healthy persons and blood samples of renal insufficient patients (i.e. patients with kidney problems), who are medicated with synthetic EPO. We tried to avoid certain blood samples with unknown parameters, such as diabetes, hepatitis, HIV, and so on, which could have an influence on the surface and structure of the erythrocytes.

In June and July 2006 we went three times for a day to the department of nephrology and kidney dialysis of the AKH to receive the healthy and EPO treated blood samples. In order to transport the blood samples we used a styrofoam container filled with ice cubes to ensure that the blood samples, anticoagulated with heparin, were not destroyed during the way back to the Vienna University of Technology.

After the standard procedure of preparation [5-8] of our blood samples (see Chapter 5) the cells were imaged with the AFM using tapping mode.

Afterwards AFM force curves were measured. The force curve parameters of the healthy and with EPO treated blood samples were finally compared.

3. Overview of Erythrocytes and EPO

The erythrocytes give the blood its red colour. Blood contains different types of cells and the so-called blood plasma (a fluid rich of proteins such as the coagulation factors). Red blood cells (erythrocytes), white blood cells (leucocytes) and platelets (thrombocytes) make the cellular part of the blood.

In this project, we investigated red blood cells of untreated patients and red blood cells of patients treated with erythropoietin and its synthetic equivalent EPO. More specifically we are using blood samples from patients with kidney failure. A dysfunction of the kidney causes a decreased production of erythropoietin. The kidneys produce the erythropoietin in the interstitial cells. Such patients get EPO as medication to antagonize a renally caused anemia.

The red blood cells carry oxygen to the periphery of the body and carbon dioxide from there back to the lung to exchange the gases and start the circle again. This is possible with the help of hemoglobin. It makes about 30 percent of the whole red blood cell. It is responsible for the adequate transport, because the CO_2 is transported in H^+ and HCO^- . For this change of the carrier substances a substance called carboanhydrasis is needed as catalytic agent [1].

This whole system is a major part of the acid/base management of the body. There are about 4.5 - 5 million red blood cells per μl blood.

Erythropoiesis [1]:

Back to the red blood cells: they develop from the proerythroblast as the ancestor cell and other different steps in between to the reticulocyte. The reticulocyte has still got the nucleus. This is the last stadium before becoming a real red blood cell. EPO supports the development of the red blood cells in the bone marrow.

In the bone marrow EPO connects with the transmembrane erythropoietin receptor on the erythroblast.

The EPO-Receptors were also detected in somatic cells (other body cells) like on neurons, astrocytes, microglia (cells of the brain and nervous system) and on cardiocytes (heart muscle cells). Among the known functions of stimulating the erythropoiesis, they are also thought to be part of the mitosis (cell division), chemotaxis (the calling of immune cells to the inflammation place), angiogenesis (growing of vessels), the apoptosis inhibition (controlled cell death), and some more responsibilities.

A major difference between red blood cells and other cells in the human body is:

- red blood cells have no nucleus.
- red blood cells have no mitochondria.

These two facts cause some problems:

Red blood cells are not able to produce any proteins (because the protein synthesis is depending on having a nucleus); e.g. they cannot synthesize membrane particles if getting damaged.

The problem of having no mitochondria (which are the “energy providers” of many cells) is that they only can work with pure glucose as substrate following the anaerobe pathway making energy out of it.

Some facts about red blood cells [2]:

Their size is about $7,5\ \mu\text{m}$ in diameter and they are about $2\ \mu\text{m}$ thick.

They are donut shaped (= biconcave).

The surface is about $135\ \mu\text{m}^2$.

Mean cell (corpuscular) volume: 90 fl

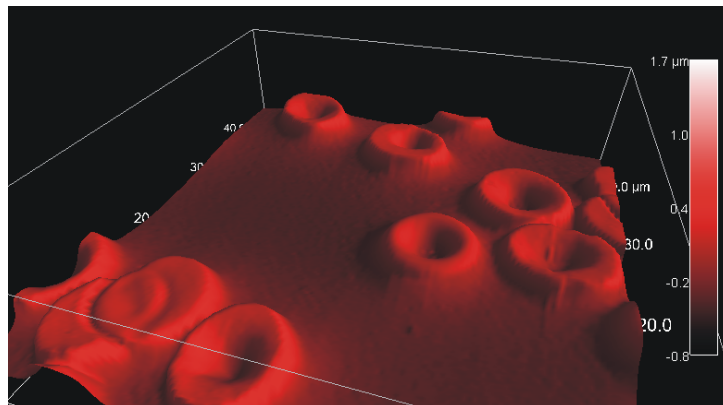


Figure 1: AFM tapping mode topography image of red blood cells on glass slide, image size $40\mu\text{m} * 40\mu\text{m}$, dried cells in air

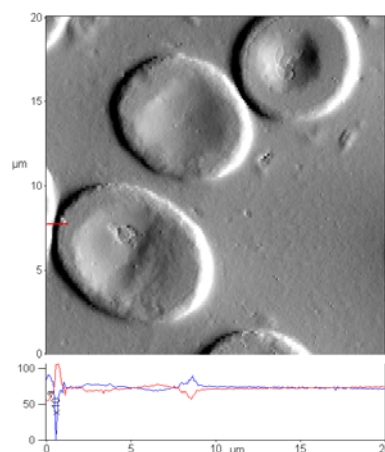


Figure 2: Height profile image of red blood cells on glass slide, image size $20\mu\text{m} * 20\mu\text{m}$, dried cells in air

4. The Atomic Force Microscope (AFM)

The Atomic Force Microscope (AFM) was invented by Binnig, Quate and Gerber in 1986[3]. It is a very high resolution type of scanning probe microscope for imaging, measuring and also manipulating matter. The main parts of the AFM are the cantilever with a sharp tip at its end, which is used to scan the sample surface, a IR-laser, which is aligned with the cantilever and a deflection sensor, which is a photodiode detecting the position of the cantilever and an inverted optical microscope. The measuring principle is based on the fact that there are very small forces ($<1\text{nN}$) between the AFM tip and the sample surface. The prepared sample, usually fixed on a glass slide in our case, is mounted on a piezoelectric tube, which makes it possible to scan the sample in x- and y- direction. There are two possibilities of doing measurements with the AFM, the contact mode (DC mode) and the dynamic mode (tapping, AC mode).

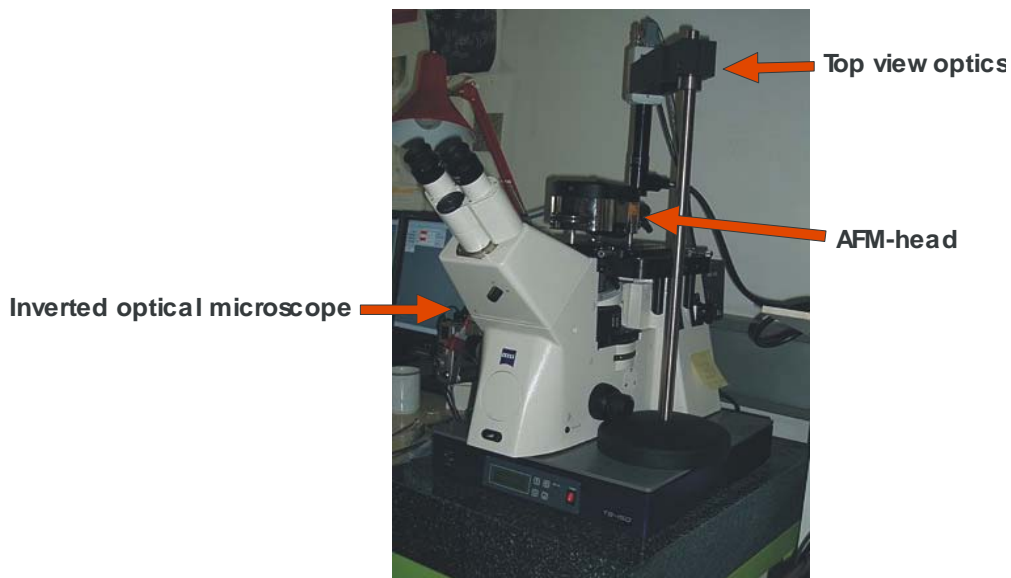


Figure 3: Optical light microscope and AFM head

4.1. Contact mode

In contact mode the cantilever tip is in mechanical contact with the surface of the sample. During the first contact, the atoms of the cantilever tip sense a repulsive force, which is caused by the overlap of the electronic orbitals of the atoms of the tip and the atoms of the sample surface. To scan the surface of the sample the tip has to be in contact with it. This causes a movement of the cantilever in z-direction and thereby a change of the deflection signal. The deflection signal is permanently sensed and compared in a DC feedback amplifier to a preset value of deflection, the setpoint[4]. If the deflection value is different from the preset value, the feedback amplifier applies a voltage to the piezo to raise or lower the cantilever tip relative to the sample surface in order to restore the preset value of deflection. So the voltage to the piezo is directly correlated to the cantilever movement and the height of the topography of the sample surface is given.

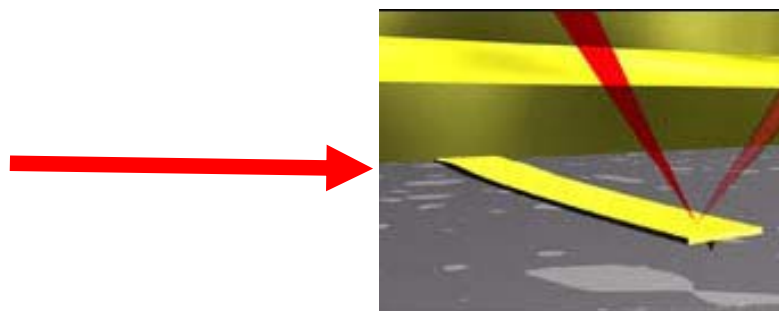


Figure 4: Laser beam reflection on the cantilever

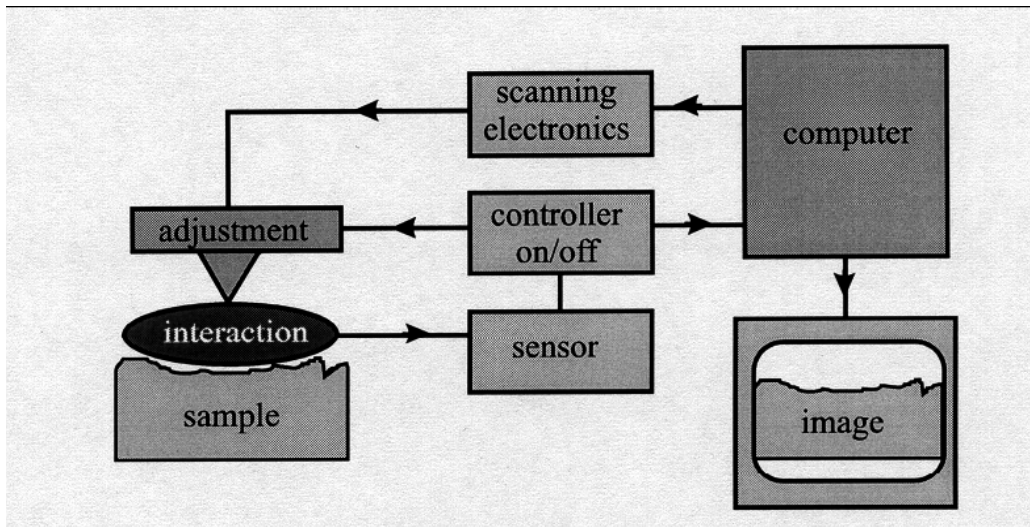


Figure 5: Feedback loop

4.2. Dynamic mode

In dynamic mode (tapping, AC mode) the distance between the tip of the cantilever and the sample surface is constant, a few femtometers. During the measurement, there is no contact between the tip and the sample surface. This technique is used to avoid damaging the sample by scratching over it. In tapping mode the cantilever is oscillated at or close to its resonance frequency[4]. The amplitude, the phase and the frequency are interacting by tip-sample interaction forces. These modifications provide information about the sample's characteristics. In contrast to contact mode, the cantilever oscillation amplitude is kept constant. The amplitude is permanently measured and a feed back loop adjusts the cantilever z-value due to the separation between the tip and the sample surface which is defined by the setpoint-amplitude. Through this process the topography of the sample surface is obtained.

4.3. Force curves

Another usage of the AFM is the measurement of force-distance curves. With these curves it is possible to observe bonds between single molecules and perform elasticity measurements of single cells. The AFM tip is approached towards and retraced from the surface, while the force between the tip and the surface of the sample is observed. The force-distance curves are a function of the z-value and the deflection signal (LVDT-signal, see below) of the cantilever [4].

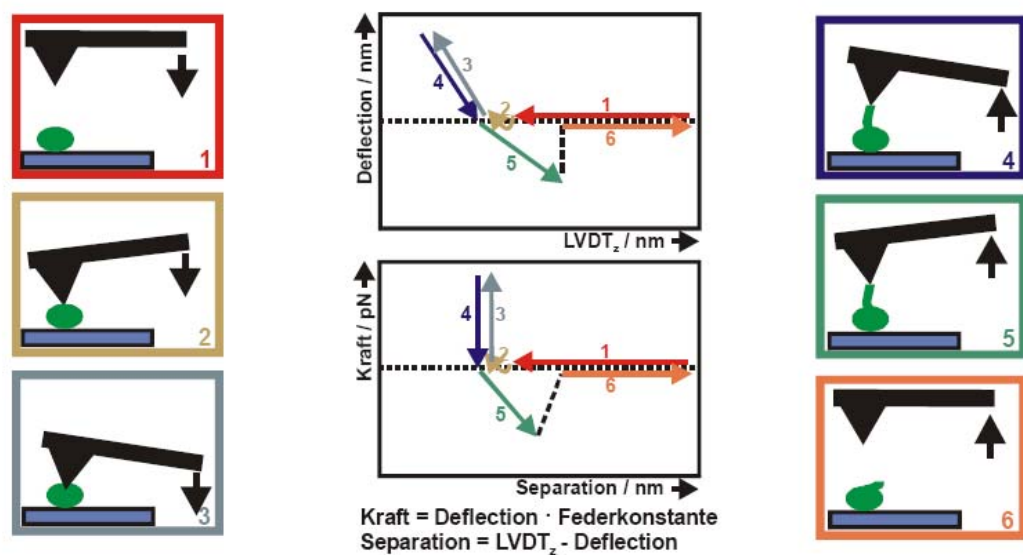


Figure 6: Principle of a force-distance curve

As seen in Figure 6 the position of the cantilever is far away from the sample surface and there is no movement of the cantilever (1), this means the deflection signal is zero. Then the AFM tip approaches the surface of the sample. Close to the contact of the tip and the sample surface, they are still not in contact (2) , there is an attractive force between the atoms of the

cantilever tip and the sample (Van-der-Waals force). Now the tip is in contact with the surface of the sample. If the z-voltage gets increased, the cantilever starts to bend and there is an increase of the deflection signal (3). After decreasing the z-voltage of the cantilever the tip retracts from the surface and the value of the deflection signal is lower than zero, the adhesion area (4). Is the adhesion force lower than the pull off force of the cantilever, the tip disengages from the sample surface (5) and the cantilever moves back to the starting position (6). With this method it is possible to detect interactions between the tip and the sample[4].

The voltage-depending expansion of the z-piezo is monitored by a Linear variable differential transformer, a type of electrical transformer used for measuring linear displacement. The LVDT-signal measures the position of the head and not the position of the cantilever tip. Therefore the parameter separation is used. The separation is the difference between the LVDT signal and the deflection (see also chapter 8).

5. Sample preparation

The blood obtained from healthy and renal insufficient patients from the department of nephrology and kidney dialysis of the AKH, anticoagulated with heparin, was attached to a glass slide. This was achieved by using poly-L-lysine solution (0,1% in water), glutaraldehyde (1% solution) and phosphate buffer saline (PBS).¹

First the glass slides were dipped in the poly-L-lysine solution, rinsed with the glutaraldehyde and then washed with the PBS. The blood was diluted in PBS 20 times and attached to the treated glass slide surface. After waiting for 5 minutes the glass slides were treated with the glutaraldehyde again. After interacting for 1 minute the blood cells were washed with PBS and the sample was mounted on the atomic force microscope [5-8] (MFP-3D, Asylum research, Santa Barbara, CA, USA).

¹ We thank Univ.Prof.Dr. Sunder-Plassmann for providing these chemicals, O.Univ.Prof. Dipl.-Ing. Dr.techn. Stachelberger for giving us information how to handle these chemicals , Univ.Prof. Dipl.-Ing. Dr.techn. Benes and Ao.Univ.Prof. Dipl.-Ing. Dr.techn. Martin Gröschl for providing their lab refrigerator for storing these chemicals.

6. Force Mapping using MFP-3D and Igor Pro

6.1. Getting started

Open the FVPanel by clicking *MFP Controls \ Force Mapping*.

Set *Points per line* and *Number of lines* for your experiment.

By choosing *Number of lines* = 1 you can create your own path.

Select one of the tracing windows and check the box beside *Show Array*.

Green points get visible in your tracing window. After clicking *Edit Array* you can manually set the points like you want.

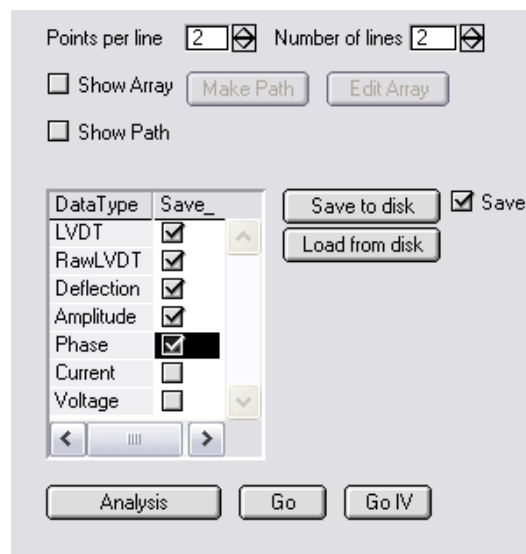


Figure 7: FVPanel

6.2. Save/Open Waves

If you want to use the same arrangement of points more than one time you can save the coordinates of your wave by using the arrays of data for x and y: FVX and FVY. First of all open the Data Browser (*Data/Data Browser*) and position the red array within the Data Browser panel (standard is on root) on FV which you can find in *packages \ MFP3D \ FV*.

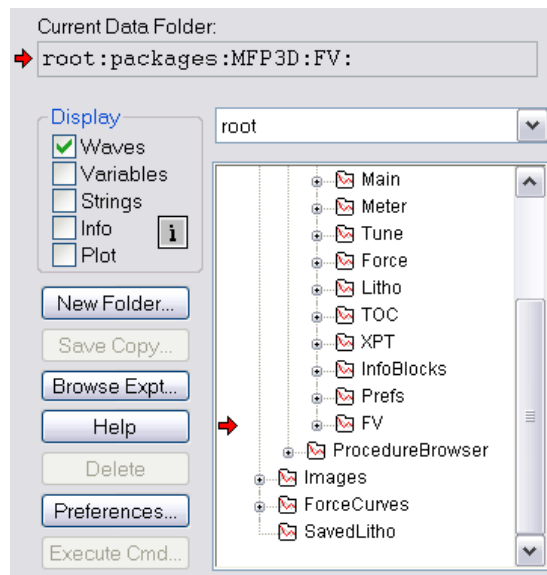


Figure 8: Data Browser

This is important for Igor pro to manipulate the wanted waves.

Then go to *Data \ Save Waves \ Save Igor Binary...*

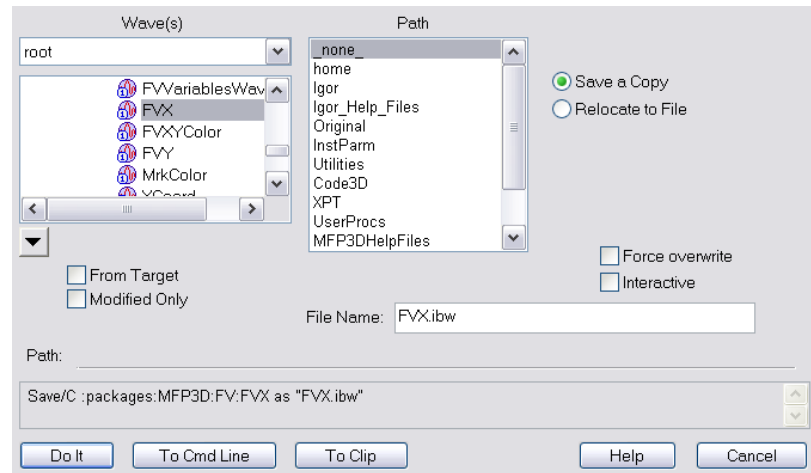


Figure 9: How to save Igor Binary

In the Waves box choose FVX and save it. Do the same steps for FVY. The loading of the FVX and FVY waves works in an analog way. Select *Data \ Load Waves \ Load Igor Binary* and choose FVX then FVY and your saved points arrangement will appear in the selected trace window.

To manipulate the FVX or FVY wave use the command panel and type e.g. $FVX = \text{some data (unit is meter)}$. You can also move the points to another position by typing e.g. $FVX += 3e-6$. If you want to manipulate only one single point you can address it by using [0] indices in brackets (Igor Pro starts counting indices with zero).

6.3. Force Mapping

Check the *Save* boxes beside the data which should be saved. Using the Force Panel within the Master Panel you can define a base suffix for your force mapping data. It is analog to the base suffix for the images.

LVDT is the linear variable differential transformer (an inductive sensor), which should be checked by doing your force curves.

You can start your measurements at the marked points by pressing the *Go* button (see Figure 7).

For saving your curves it is important to check the box beside *Save*. You can also manually save curves by clicking on *Save to disk* (only current curves). For both ways you need to select a folder for saving.

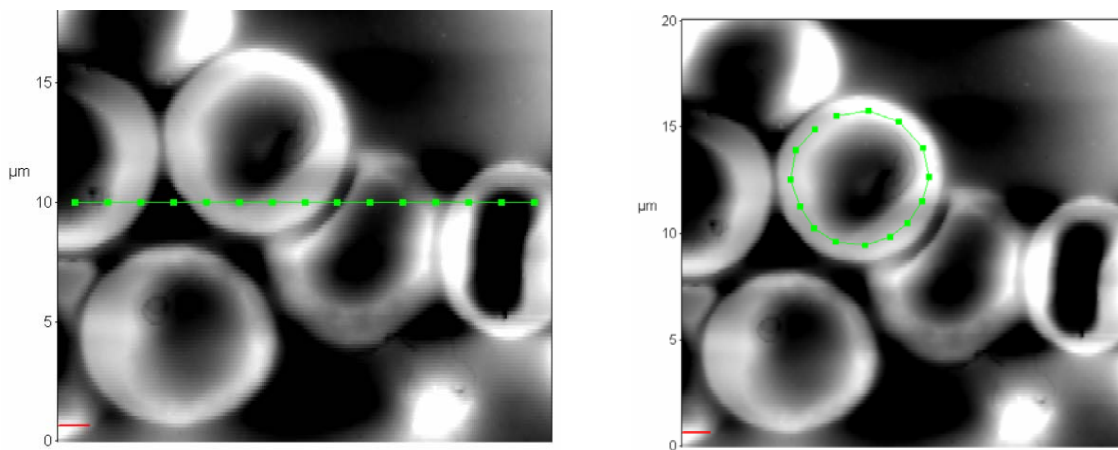


Figure 10: Force mapping: left: along points given on a line; right: on a user-given path, here circumventing a red blood cell; top trace: red blood cell topography; bottom trace: height profile along line.

6.4. Analysis

For analyzing the saved data, go to *Load from disk* and select the correct directory. Now select the right ensemble of curves (base name and suffix).

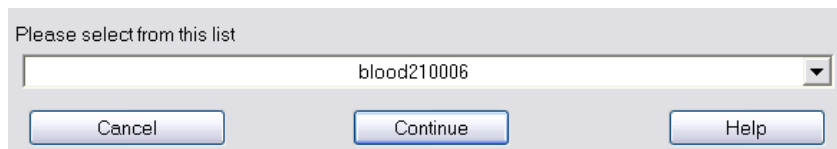


Figure 11: Load from disc

In this case base name is blood21 and suffix 0006. Clicking *Continue* loads the ensemble of waves for showing the force curves again, or for analyzing the saved data.

Now choose *Analysis* within the FVPanel (see Figure 7). FVAnaPanel is popping up.

Check the functions in the Offline column you want to have analyzed. Alternatively you can select *All* (when you want to use all the data)

Clicking on *Run Offline* is processing the data.

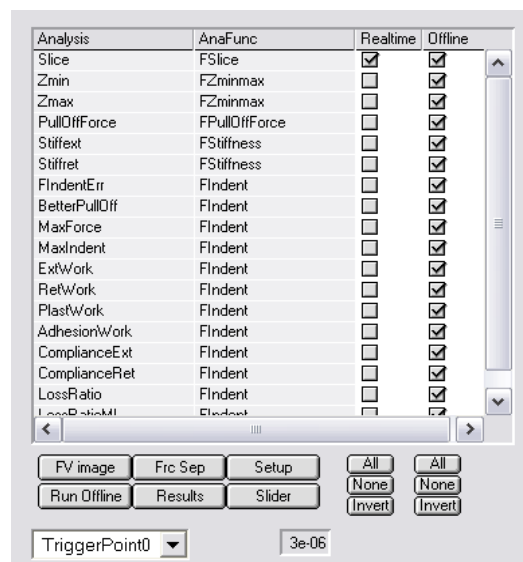


Figure 12: Analysis panel

Force vs. Separation shows the force in x-scale and the separation which is

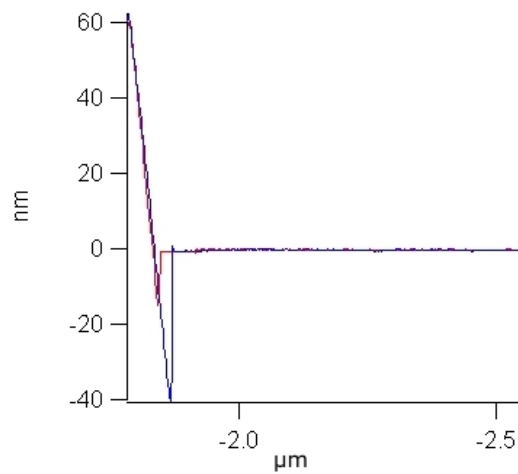


Figure 14: Typical force curve (explained in chapter 4.3. Force curves)

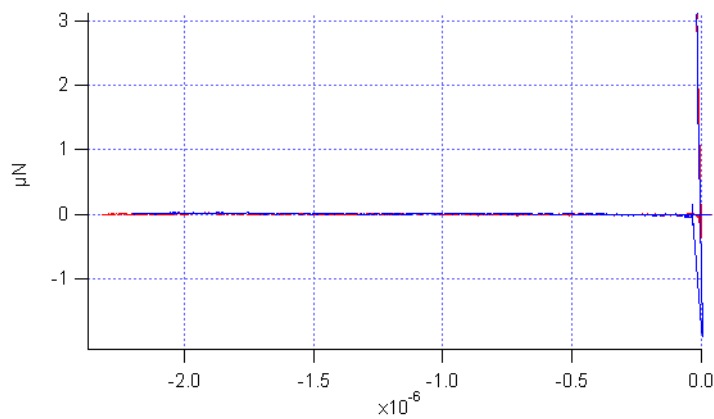


Figure 15: Force vs. Separation curve (explained in chapter 4.3. Force curves)

FV Image: Choose a result function like Zmin in this example picture. By clicking on the stripes you can select the single force curves and data.

6.5. Save Analysis Data

You can use and save the data shown in results by copy and paste to Excel.

If you want to use all data of your point ensemble you can save the results to a text file by:

- Red array to FV in Data Browser
- Data \ Save Waves \ Save Delimited Text...
- Select FVResults in the left box
- Click “Do It”

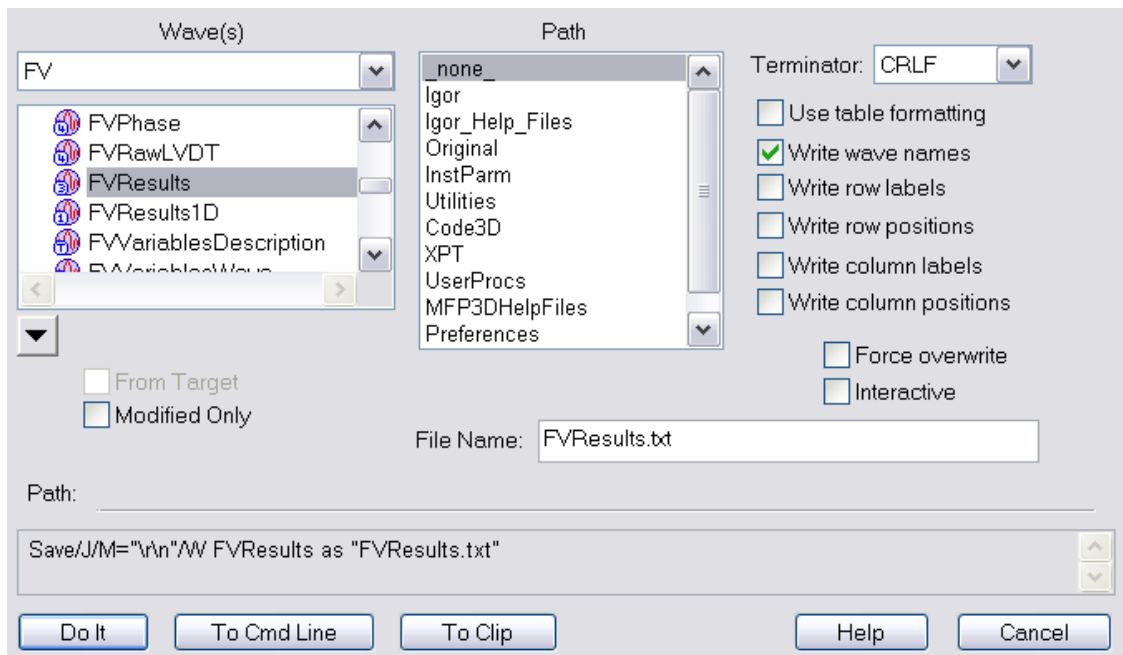


Figure 16: How to save the results

7. Measurement

The images were recorded by using a cantilever with 70 kHz resonance frequency in air. We used tapping mode and scanned with 0.43 Hz (i.e. a little bit less than two lines per second). The image size was $20 \times 20 \mu\text{m}^2$. After imaging the erythrocytes we selected each single cell and created the force mapping path, as explained in chapter 6. We created a path with 15 points and triggered the force, which is exerted to the tip to move into the cell to 3 μN . Force curves as shown in chapter 6 (LVDT vs. Force) and Force vs. Separation curves were recorded. In some of our 15 curves ensembles we found that the first was no real force curve because the tip sample distance was often too large to reach the sample and with it the wanted trigger point. In these cases we excluded the respective first curves. One of the measured parameters is the PullOffForce, which indicates the stickiness of the red blood cells.

To calibrate our system we performed a force curve on a hard glass surface and set the DefInOLS (deflection inverse optical lever sensitivity) accordingly to get the real height of the erythrocytes as well as real Force vs. Separation curves. At the edges of the cells we obtained small artifacts due to the feed back mechanism of the system.

After recording enough representative force curve parameters, we exported our results to Excel in order to compare the relevant parameters. Mean value and standard deviation were calculated for each sample as well as for the whole amount of healthy and EPO blood.

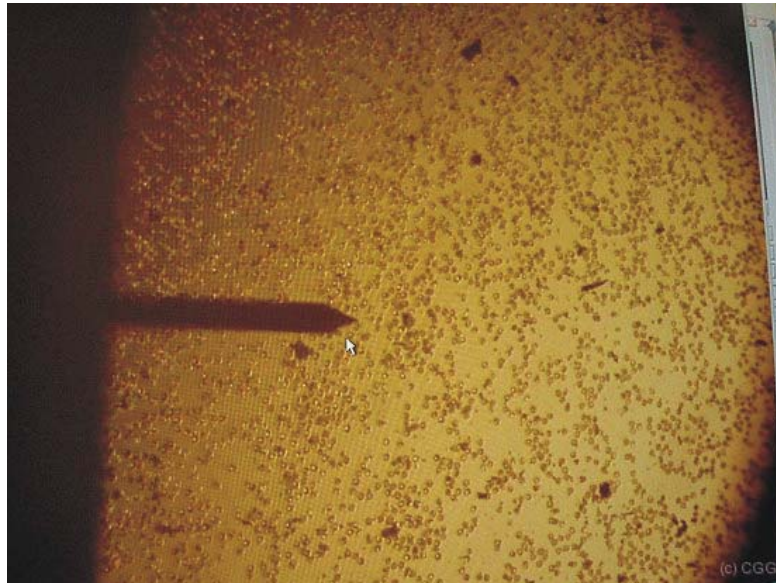


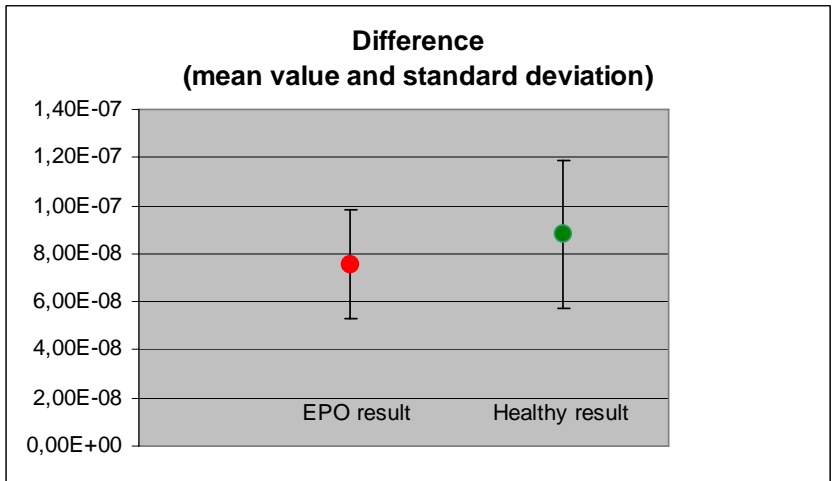
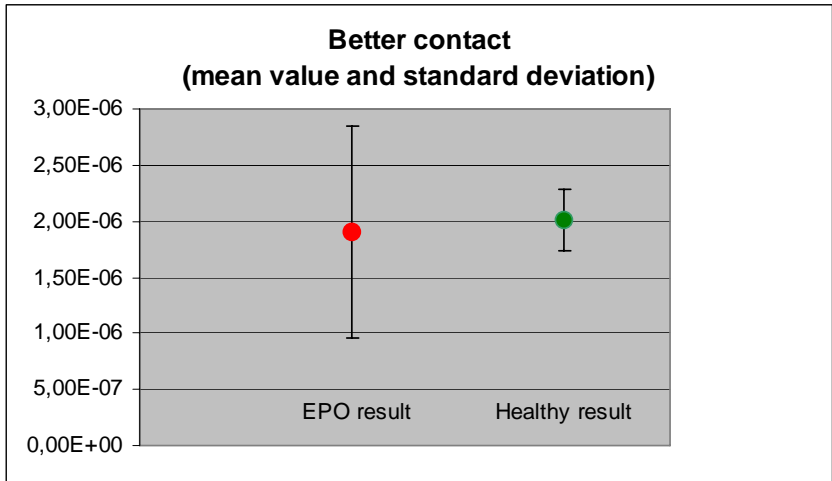
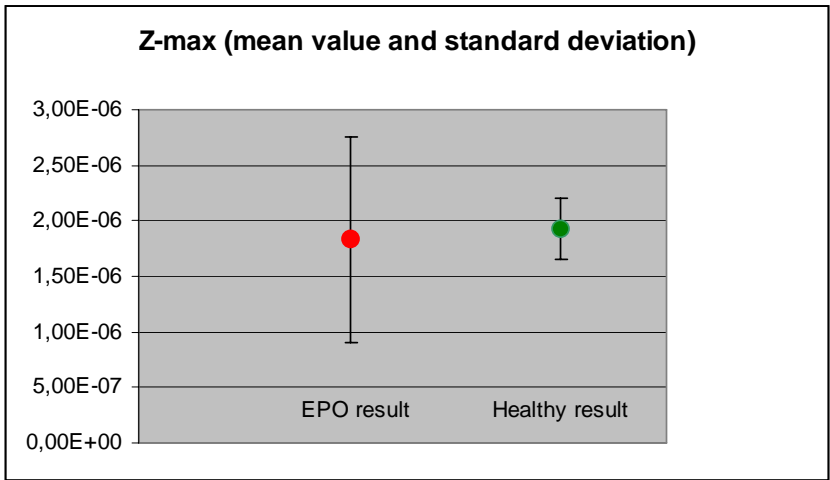
Figure 17: Scanning tip over red blood cells. The diameter of the red blood cell is 6-8 μ m

8. Results

According to our question how deep we can invade the red blood cells with the tip (with a triggered force of 3 μN) we measured 26 blood samples (14 EPO patients, 12 healthy people) with about 200 force curves per sample, did our analysis and obtained the following results (the parameters are explained on p.25 ff):

PARAMETER	EPO results	Healthy results	Difference of mean values
Zmin	mean value	mean value	
	4,16442E-06	4,28059E-06	
	standard deviation	standard deviation	
	9,87909E-07	2,81105E-07	1,16179E-07
Zmax	mean value	mean value	
	1,82976E-06	1,92574E-06	
	standard deviation	standard deviation	
	9,31605E-07	2,74918E-07	9,59844E-08
PullOffForce	mean value	mean value	
	1,31067E-06	1,69523E-06	
	standard deviation	standard deviation	
	6,36957E-07	8,00108E-07	3,84566E-07
BetterPullOff	mean value	mean value	
	1,27647E-06	1,69388E-06	
	standard deviation	standard deviation	
	6,37819E-07	7,65965E-07	4,17415E-07
MaxForce	mean value	mean value	
	3,10041E-06	3,04527E-06	
	standard deviation	standard deviation	
	4,60978E-08	7,17875E-08	5,51466E-08
BetterContact	mean value	mean value	
	1,906E-06	2,01412E-06	
	standard deviation	standard deviation	
	9,46216E-07	2,74389E-07	1,08117E-07

PARAMETER	EPO results	Healthy results	Difference of mean values
ExtWork	mean value	mean value	
	1,71672E-14	2,40466E-14	
	standard deviation	standard deviation	
	2,8724E-14	3,42159E-14	6,87945E-15
RetWork	mean value	mean value	
	1,15335E-14	2,78668E-14	
	standard deviation	standard deviation	
	2,56554E-14	3,85603E-14	1,63333E-14
PlastWork	mean value	mean value	
	5,63366E-15	-3,82023E-15	
	standard deviation	standard deviation	
	1,14748E-14	6,90447E-15	9,45389E-15
AdhesionWork	mean value	mean value	
	6,17799E-14	1,01725E-13	
	standard deviation	standard deviation	
	5,49515E-14	7,64689E-14	3,99449E-14
ComplianceExt	mean value	mean value	
	0,001194106	0,002773881	
	standard deviation	standard deviation	
	0,005697014	0,007208185	0,001579775
ComplianceRet	mean value	mean value	
	0,002273247	0,005841257	
	standard deviation	standard deviation	
	0,005063954	0,007713106	0,00356801
Difference	mean value	mean value	
	7,58076E-08	8,82612E-08	
	standard deviation	standard deviation	
	2,2809E-08	3,07747E-08	1,24536E-08



Note:

- LVDT (Linear variable differential transformer is a type of electrical transformer used for measuring linear displacement. Current is driven through the primary coil at A, causing an induction current to be generated through the secondary coils at B.) Displacing the core in one direction, the voltage in one coil increases as the other decreases, causing the output voltage to increase from zero to a maximum. The magnitude of the output is linear to the displacement of the core, the phase is indicating the direction of the displacement. An advantage of the LVDT is that the core is not touching the inside of the tube, so LVDTs are often used for position feedback.

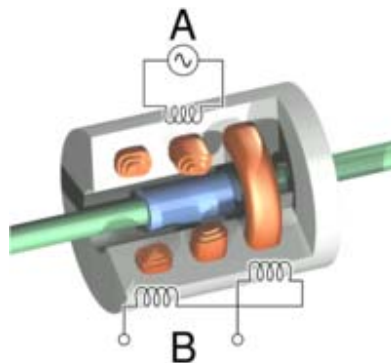


Figure 18: Cutaway view of a LVDT

The AFM is using a photodiode detecting the deflection of a laser spot, which is reflected by the scanning tip to get its LVDT signal. This modern kind of “LVDT” is able to detect tiniest deflection. It is sensitive enough to digitalize the movement of the scanning tip in nanoscale.

- Zmin: Zmin is the minimal movement of the Z-sensor.
We do not use Zmin in our results because of the different start positions of the tip.
- Zmax: is the maximal movement of the Z-sensor and the maximal approach of the triggerpoint.
- PullOffForce: is the force needed, to drop out of the sample surface.
- BetterPullOff: is the PullOffForce after baseline subtraction.
The baseline is in reality not a line because of movement of the deflection, change in temperature, noise etc.
- ExtWork: is the area underneath the extend curve.
- RetWork: is the area underneath the retract curve.
Difference of both is the plastic deformation.
- AdhesionWork: is the area under the adhesion peak.
- Compliance: is the inverted grade of the force vs. separation curve.
- BetterContact: is the Z-value at the beginning of triggering.
- Difference: is the difference between BetterContact and Zmax
=penetration depth

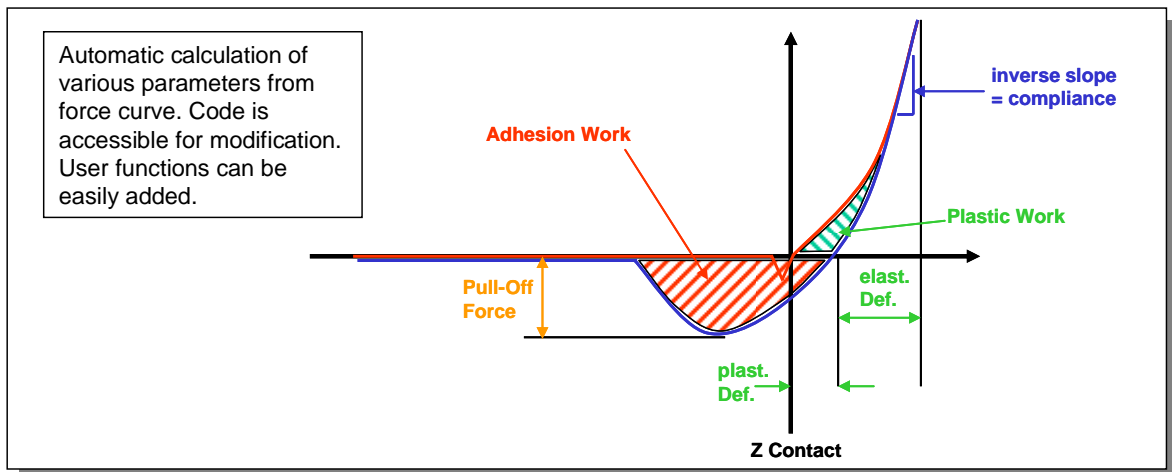
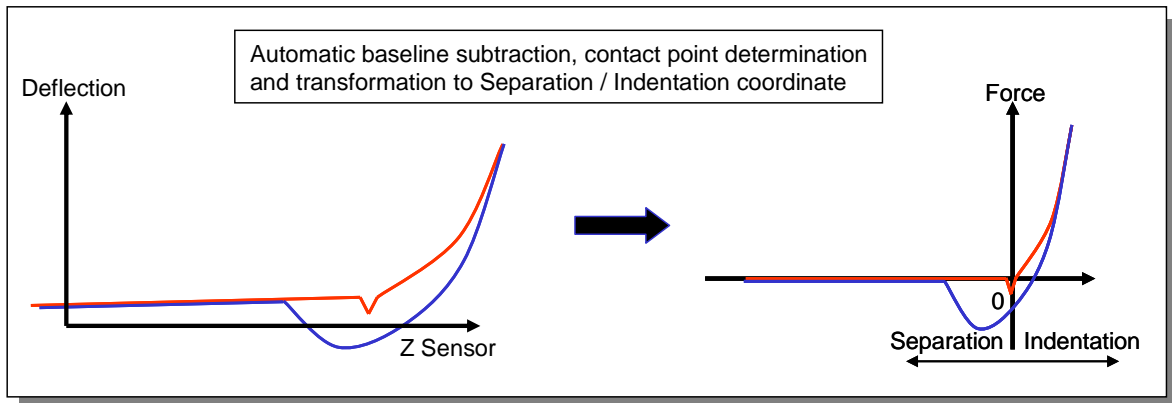


Figure 19: Force curve explanation

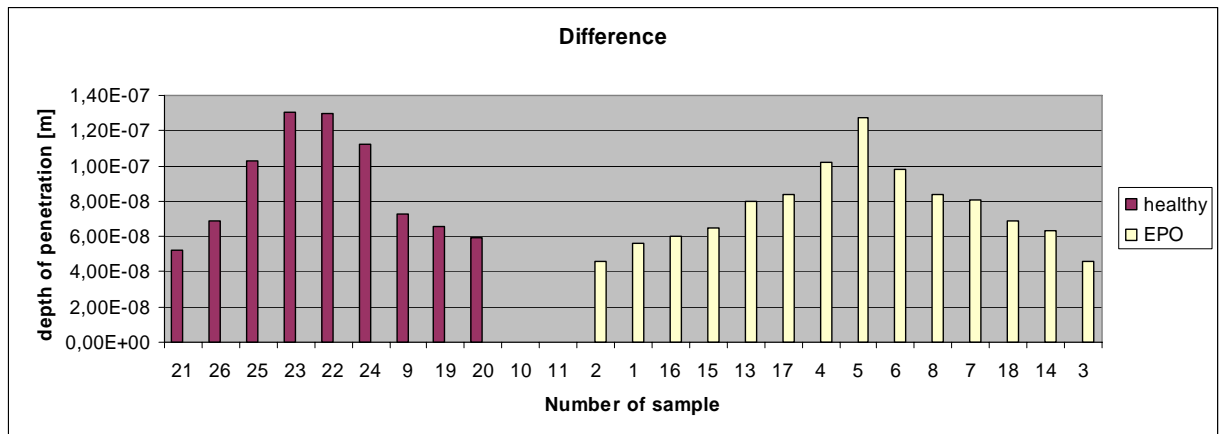


Figure 20: Mean value of the Difference of each sample

For our question the maximal movement of the z-sensor, the z-value at the beginning of triggering and the penetration depth were the relevant parameters. After averaging and calculation of the standard deviation of the values of the healthy and EPO blood samples we came to the result, that the difference of healthy and EPO red blood cells is very small and not statistically significant.

9. Discussion and Outlook

Due to the fact, that this work is just a pilot project, we think that there still could be a significant difference in the consistence of red blood cells of healthy and EPO patients. Because of the limited time we weren't able to measure more blood samples in order to find significant differences.

As a result of the AKH doctors' interest in our work and technical possibilities with the AFM for medical research we consider to intensify our collaboration on similar projects in future.

In sample 10 and 11 we found abnormalities of the red blood cells which should be examined in more detail. We found erythrocytes in this blood sample, which have not the typical donut shaped form. These blood cells were oblate and very flat. To be sure that we did no preparation failure or that the blood sample has been not destroyed during transportation, we got a second blood donation, the patient was not medicated with EPO. Studying this sample confirmed our previous results. We excluded the samples 10, 11 with these abnormalities from our results. After talking to the doctors we suppose that this abnormality could be caused by a kind of diabetes.

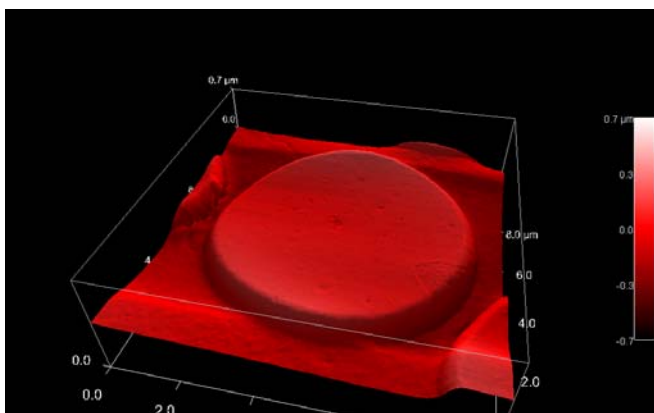


Figure 21: Blood cell abnormality (sample 11)

10. Acknowledgements

We would like to thank Dr. Ille Gebeshuber for giving us the chance to work with the AFM and for the guidance through this project.

Many thanks to Univ.Prof.Dr. Sunder-Plassmann (Meduni Wien) for providing us with the chemicals and his advice.

Many thanks to Dr. Demetriou for his assistance in obtaining healthy and EPO blood samples.

We thank the 26 anonymous patients who provided us with a blood donation.

11. References

[1] H. Lippert (2006) Lehrbuch Anatomie, Urban & Fischer bei Elsevier.

[2] Red blood cells

Wikipedia

www.wikipedia.org

[3] AFM

Wikipedia

www.wikipedia.org

[4] S. Roes (2004) Rasterkraftmikroskopische Untersuchungen von Membraneigenschaften und Membran-Protein-Wechselwirkungen, Dissertation der Mathematisch-Naturwissenschaftlichen Fakultät der Christian-Albrechts-Universität zu Kiel, Kiel, Germany.

[5] R. Nowakowski, P. Luckham and P. Winlove (2001) Imaging erythrocytes under physiological conditions by atomic force microscopy, *Biochim. Biophys. Acta* 1514, pp. 170-176.

[6] R. Nowakowski and P. Luckham (2002) Imaging the surface details of red blood cells with atomic force microscopy, *Surf. Interf. Anal.* 33, pp. 118–121.

[7] M. Lekka, M. Fornal, G. Pyka-Fosciak, K. Lebed, B. Wizner, T. Grodzicki and J. Styczen (2005) Erythrocyte stiffness probed using atomic force microscope, *Biorheol.* 42(4), pp. 307-317.

[8] F. Lui, J. Burgess, H. Mizukami and A. Ostafin (2003) Sample preparation and imaging of erythrocyte cytoskeleton with the atomic force microscope, *Cell Biochem. Biophys.* 38(3), 251-270

[9] M. Oberringer, W. Metzger and U. Hartmann (2002) Beispiele nanobiotechnologischer Ansätze in Grundlagenforschung und industrieller Anwendung, Magazin Forschung 2/2002, pp. 6-13.

[10] Biological Sample Preparation
Support Note 331 Revision A

  , www.veeco.com

12 Examples of blood sample measurements

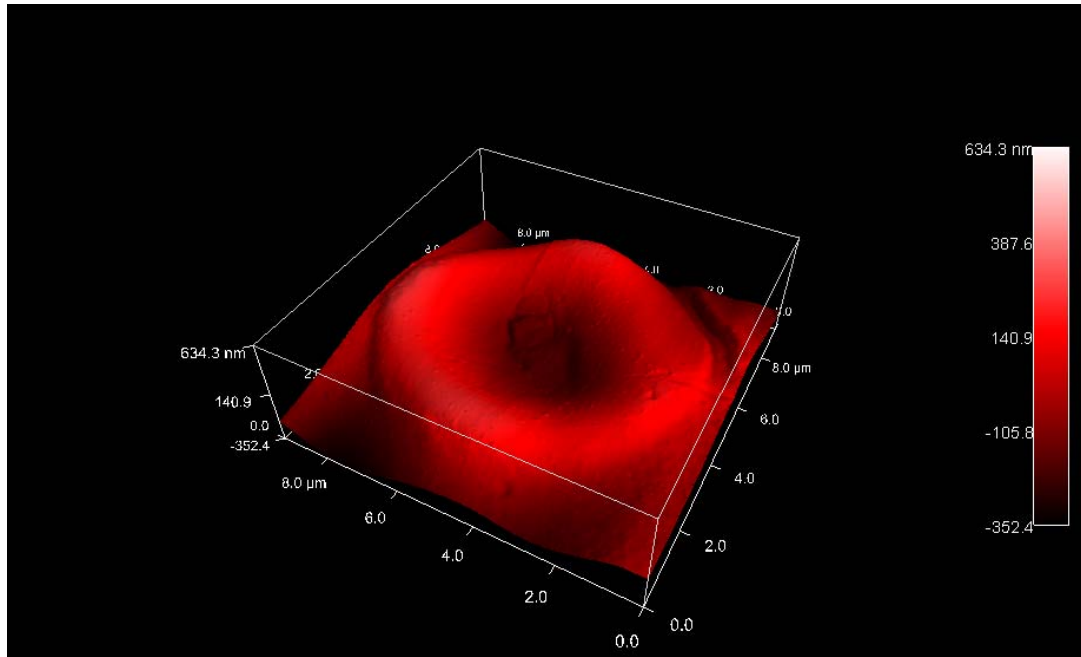


Figure 22: Blood sample 21, scan size 9*9μm²

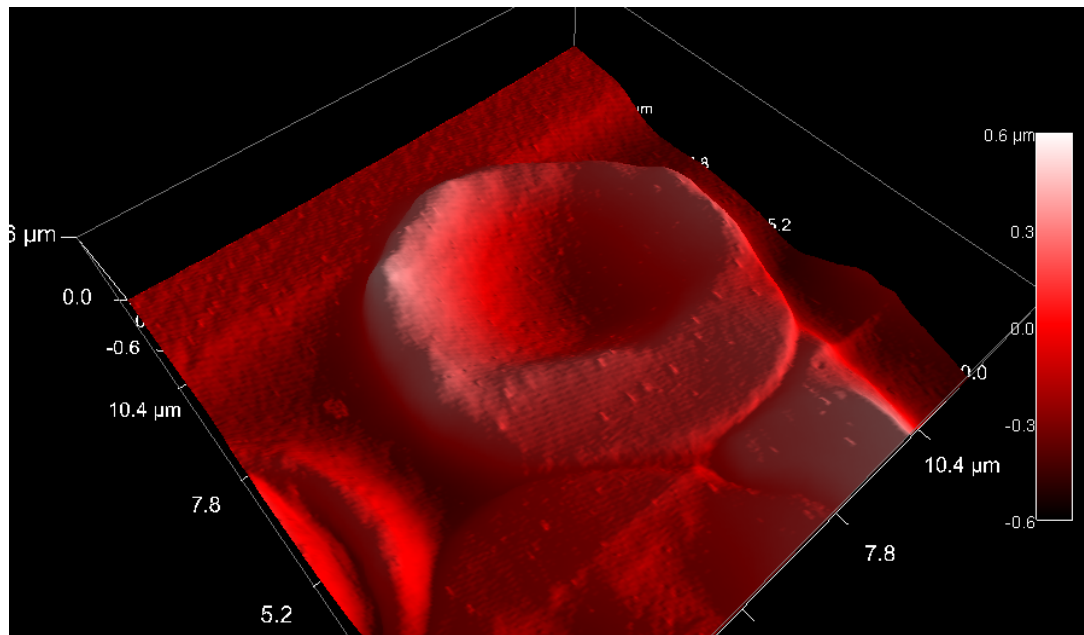


Figure 23: Blood sample 21, scan size 11, 8*11, 8μm²

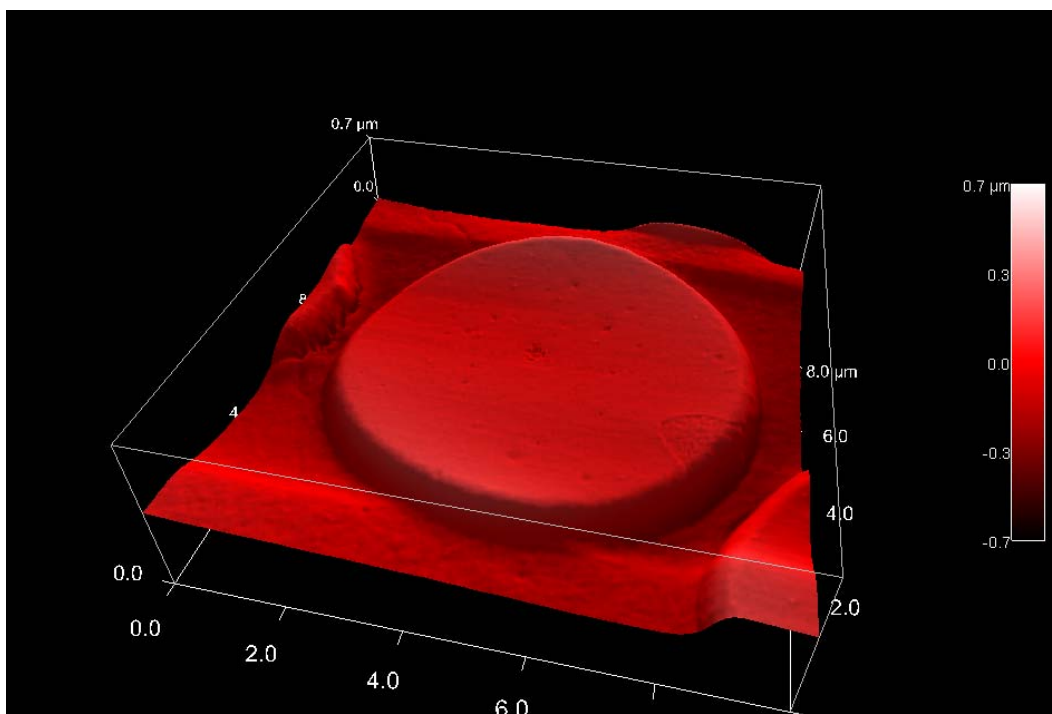


Figure 24: Blood sample 11, scan size $8 \times 8 \mu\text{m}^2$

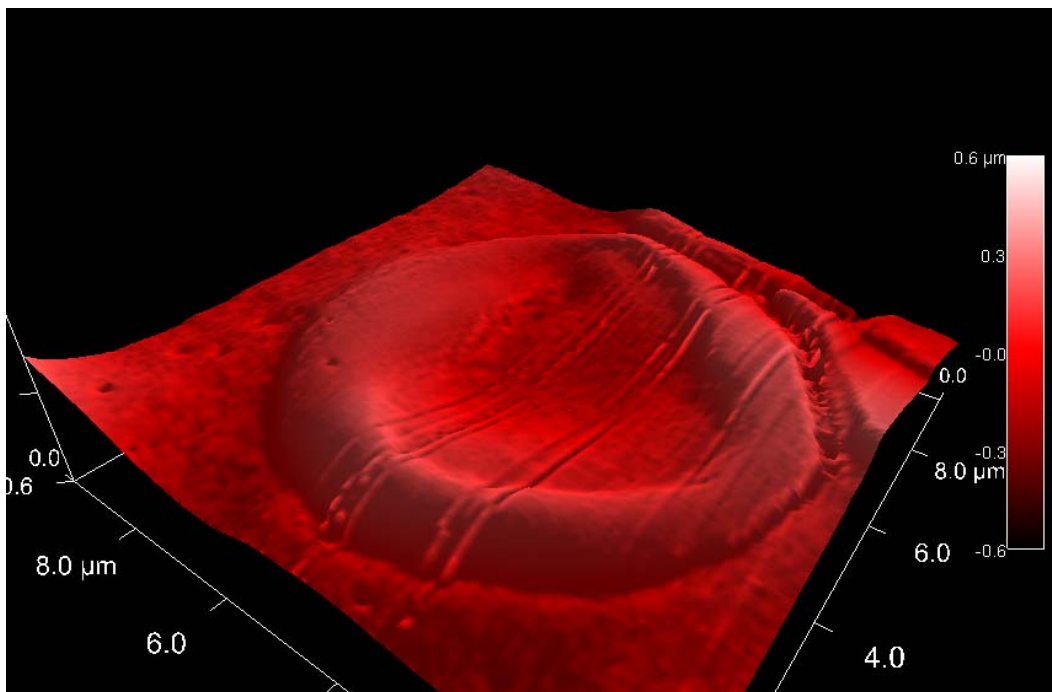


Figure 25: Blood sample 19, scan size $9 \times 9 \mu\text{m}^2$

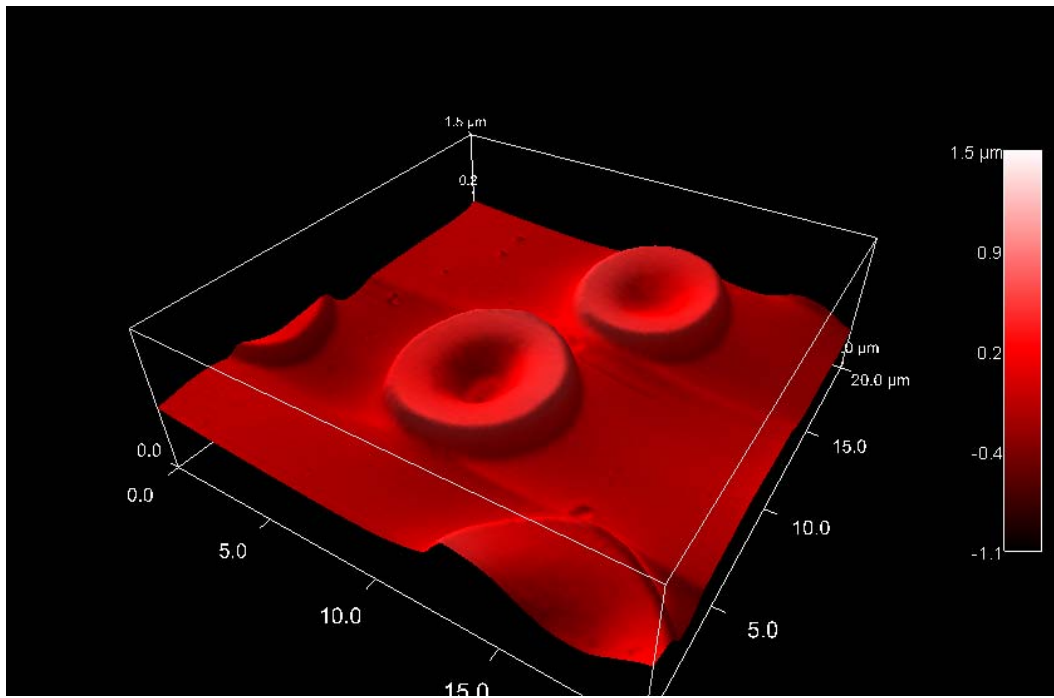


Figure 26: Blood sample 19, scan size $20 \times 20 \mu\text{m}^2$

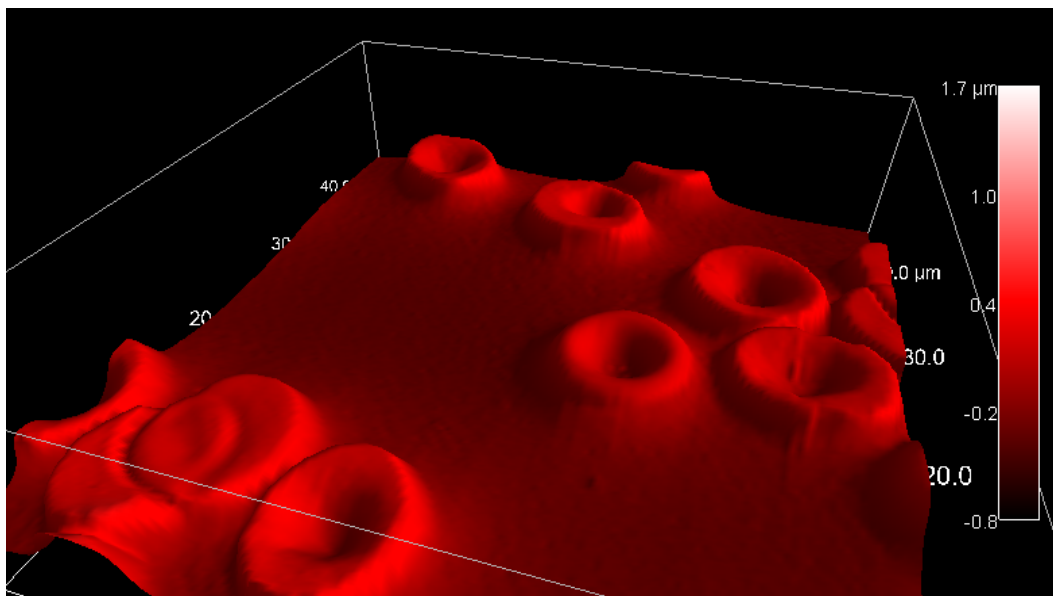


Figure 27: Blood sample 25, scan size $40 \times 40 \mu\text{m}^2$

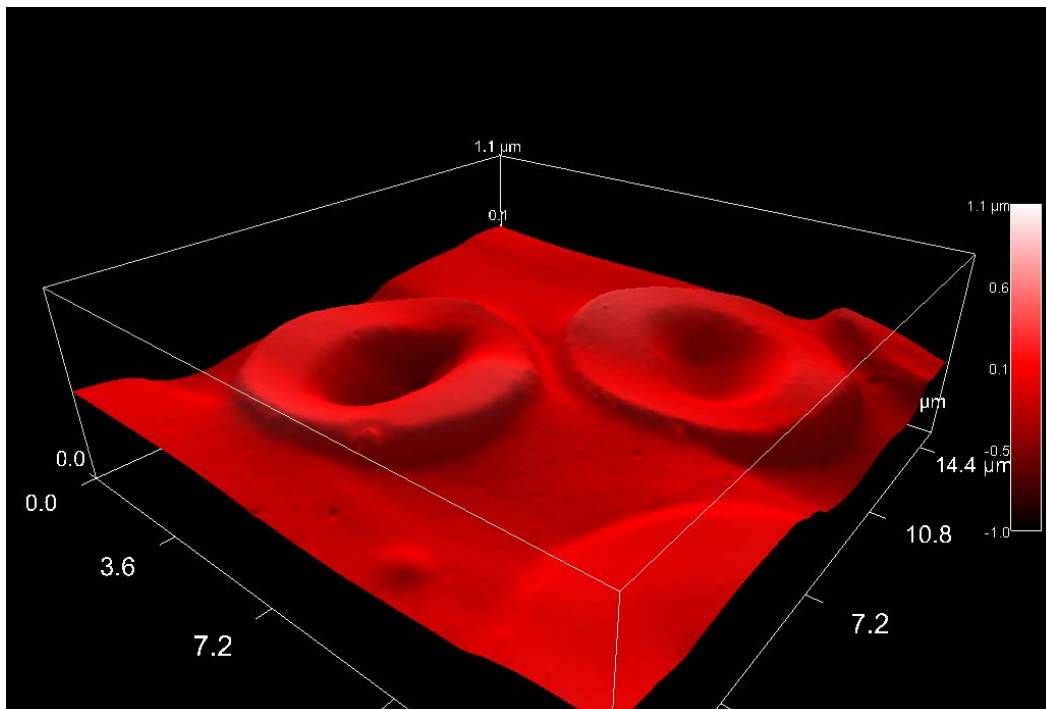


Figure 28: Blood sample 20, scan size $15 \times 15 \mu\text{m}^2$

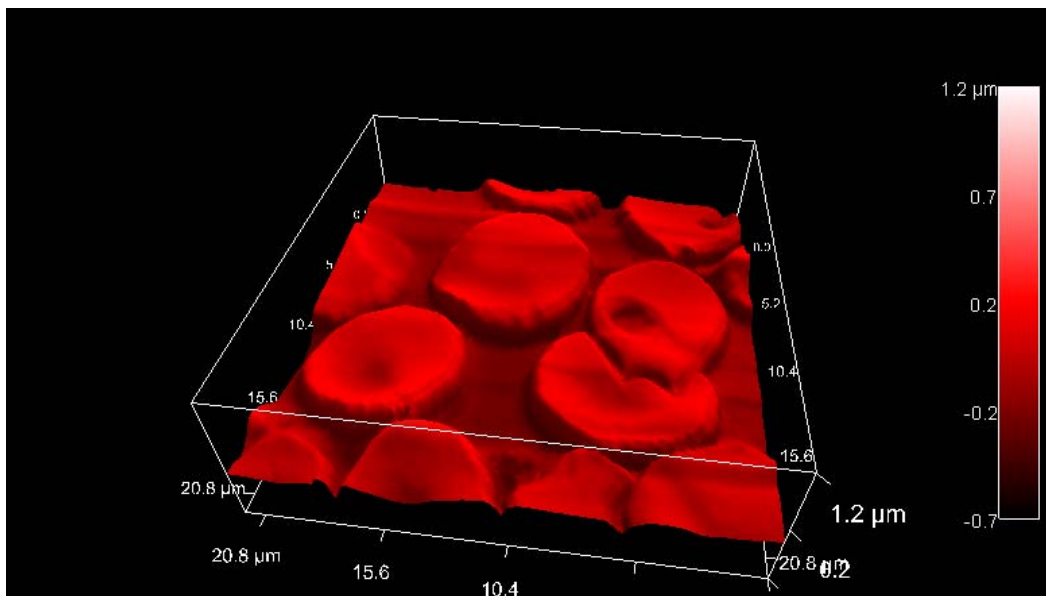


Figure 29: Blood sample 26, scan size $21,6 \times 21,6 \mu\text{m}^2$

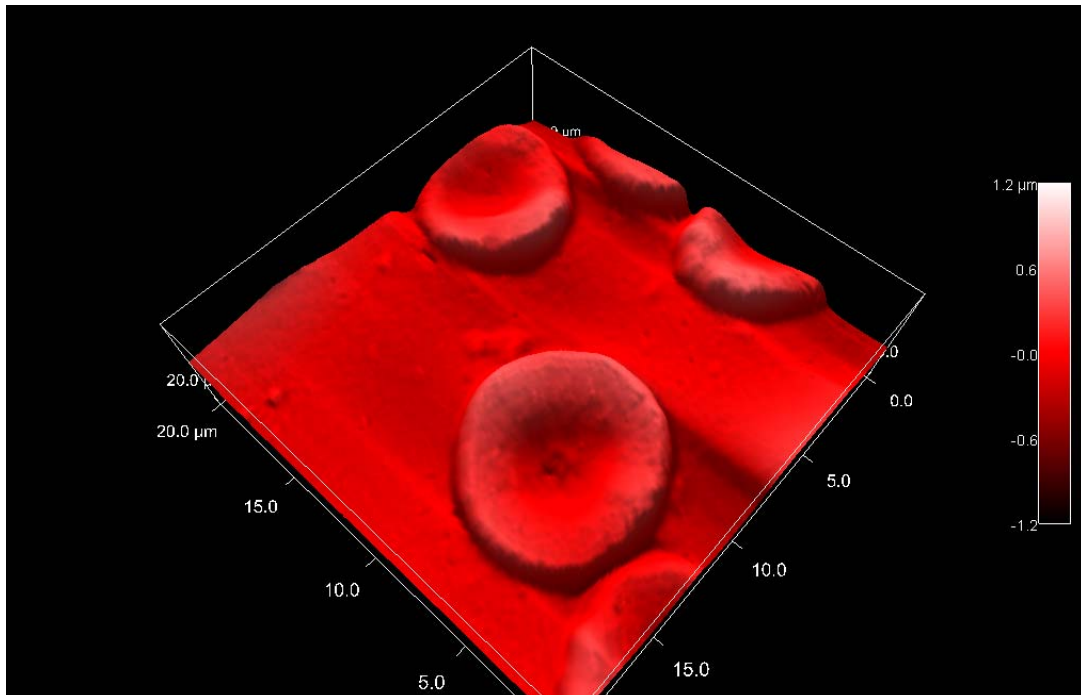


Figure 30: Blood sample 23, scan size $20*20\mu\text{m}^2$

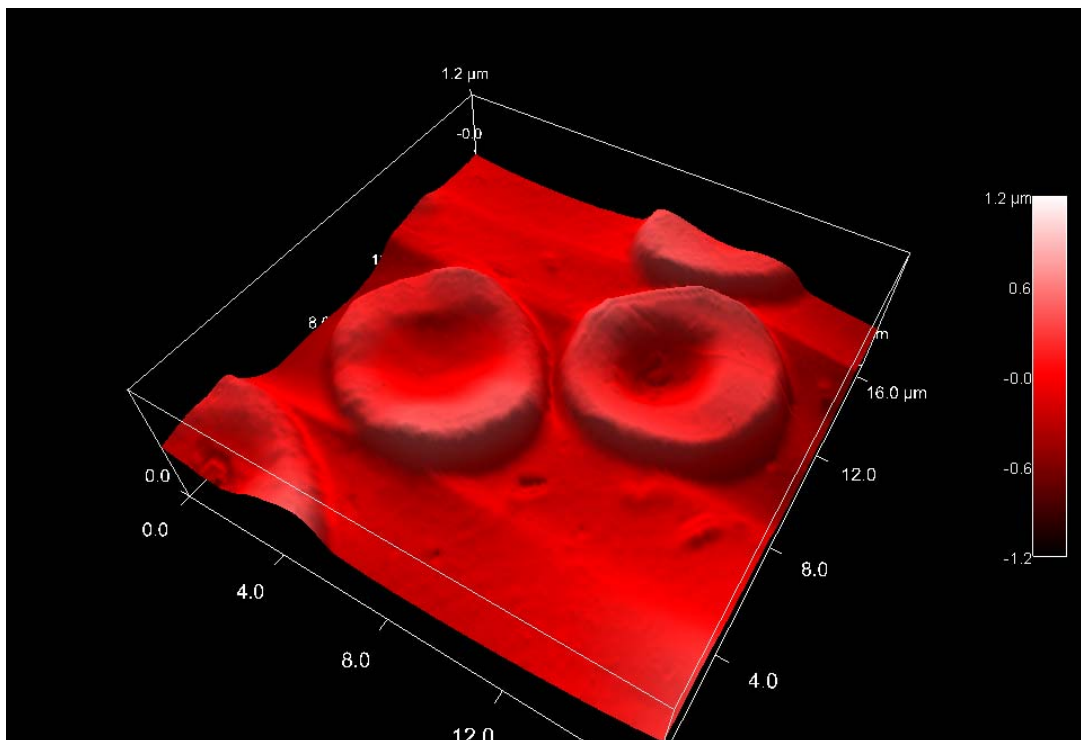


Figure 31: Blood sample, scan size $16*16\mu\text{m}^2$

Additionally to the red blood cells, some white blood cells we imaged:

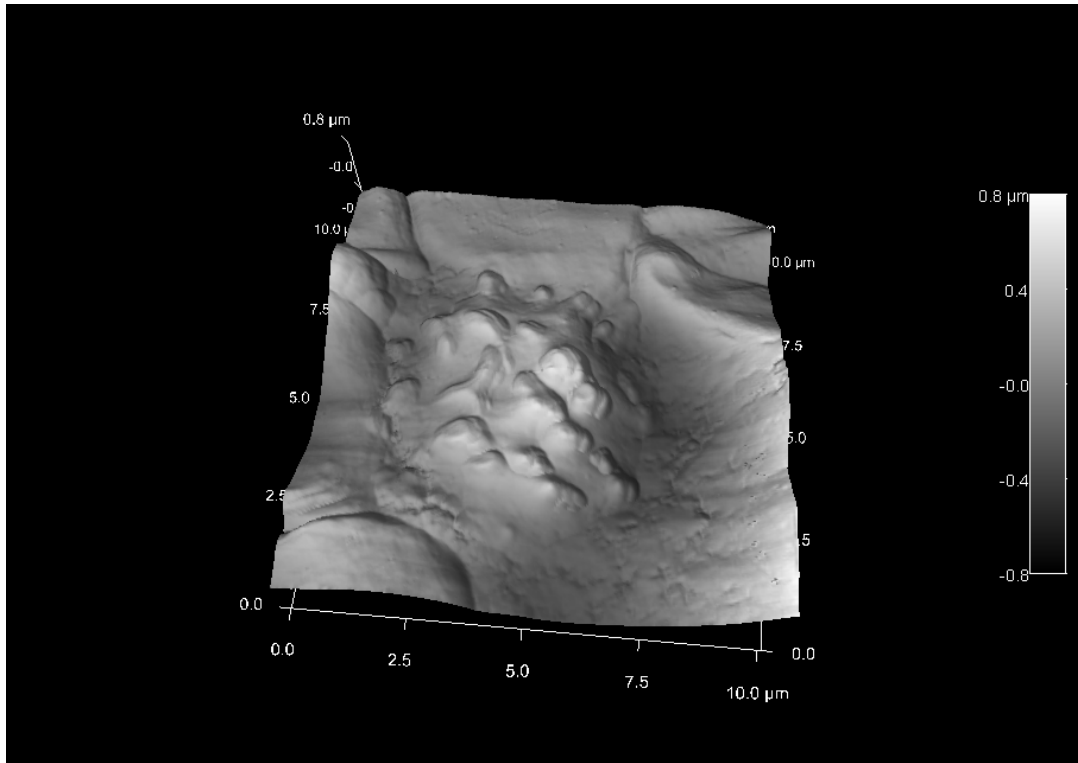


Figure 32: Blood sample??, scan size $10 \times 10 \mu\text{m}^2$

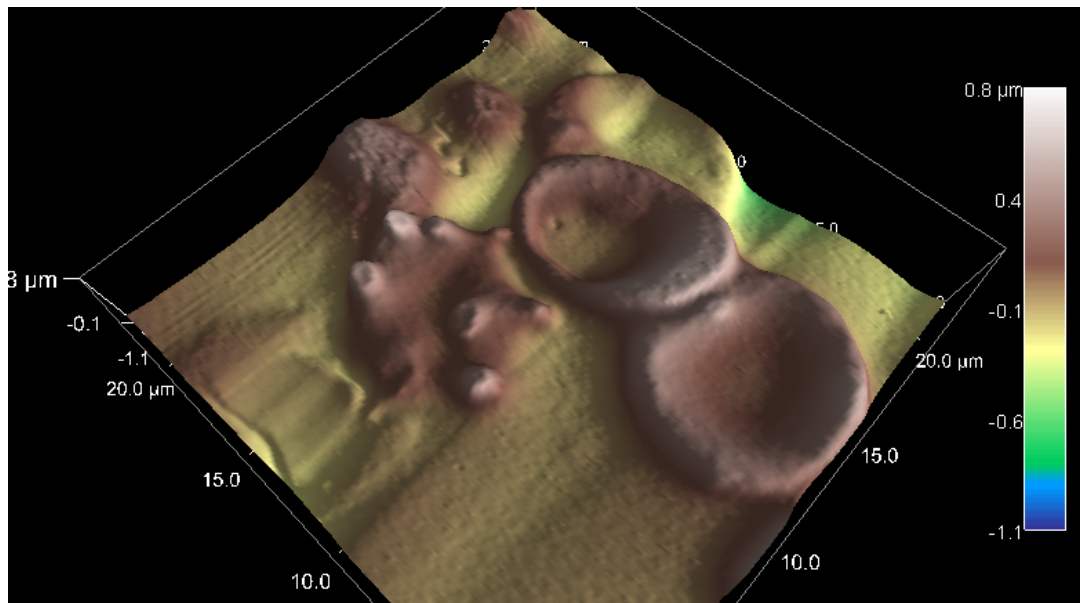


Figure 33: Blood sample 21: scan size $20 \times 20 \mu\text{m}^2$