



JAGIELLONIAN UNIVERSITY  
IN KRAKÓW

***The study of endothelial cells  
by means of atomic force microscopy***

Doctoral dissertation by  
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2022

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### Oświadczenie

Ja, niżej podpisana, Magdalena Giergiel (nr indeksu: 1039751), doktorantka Wydziału Fizyki, Astronomii i Informatyki Stosowanej Uniwersytetu Jagiellońskiego, oświadczam, że przedłożona przeze mnie rozprawa doktorska pt. „Badanie komórek śródbłónka metodami mikroskopii sił atomowych” jest oryginalna i przedstawia wyniki badań wykonanych przeze mnie osobiście, pod kierunkiem prof. dr. hab. Jerzego Koniora oraz dr. Bartłomieja Zapotocznego. Pracę napisałam samodzielnie.

Oświadczam, że moja rozprawa doktorska została opracowana zgodnie z Ustawą o prawie autorskim i prawach pokrewnych z dnia 4 lutego 1994 r. (Dziennik Ustaw 1994 nr 24 poz. 83 wraz z późniejszymi zmianami).

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## Streszczenie

W pracy badam właściwości komórek śródbłónka przy użyciu mikroskopu sił atomowych (AFM) oraz analizuję i interpretuję uzyskane wyniki. W szczególności używam metody spektroskopii sił do określenia elastyczności oraz struktury komórek. Zmiany tych cech związane są często ze zmianami funkcjonalnymi lub patologicznymi komórki.

Ważnym elementem w zaprezentowanej analizie jest interpretacja danych eksperymentalnych. Dwa najczęściej stosowane w badaniach komórkowych modele elastyczności, wprowadzone przez Hertza i Sneddona, to modele jednoparametrowe, które spełniają silne założenia, m.in. o jednorodności materiału, małych odkształceniach, specyficznej geometrii sondy itp. Układy biologiczne o złożonej strukturze, takie jak komórki śródbłónka, są dalekie od jednorodności. Analiza pomiarów krzywych siła-odległość ujawnia zmiany lokalnej sprężystości wraz z głębokością indentacji. W konsekwencji modele wieloparametrowe są niezbędne do prawidłowej analizy danych.

Zaproponowanym rozwiązaniem problemu jest użycie modelu materiału warstwowego pierwotnie zaproponowanego przez Kovaleva do opisu elastyczności cienkich warstw. Model ten został zmodyfikowany w celu przystosowania go danych zebranych dla komórek śródbłónka. Otrzymany w wyniku analizy parametr elastyczności jest opisywany funkcją schodkową ze strefami przejściowymi, w których występuje gradient elastyczności.

Drugim tematem badawczym było ilościowe opisanie struktury powierzchni błony komórek śródbłónka zatok wątroby (LSEC). W tym celu szczególnie użyteczną metodą okazało się zastosowanie algorytmów opartych na uczeniu maszynowym. W wyniku takiego podejścia przeprowadzono skuteczną analizę struktur na obrazach topograficznych z AFM.

Okazuje się, że dokładność zaproponowanej metody nie tylko przewyższa wcześniejszą analizę ręczną, ale też ten sposób jest znacznie szybszy, niewrażliwy na kontrast obrazu i w pełni deterministyczny, dlatego wyniki są bardziej wiarygodne. Ponadto przedstawiony schemat można łatwo modyfikować do różnych obiektów zainteresowania, co sprzyja wykorzystaniu sieci neuronowych jako uniwersalnego narzędzia do analizy wielu rodzajów obrazów mikroskopowych.

## Abstract

In this work, I investigate the properties of endothelial cells using the atomic force microscope (AFM), as well as analyse and interpret the obtained results. In particular, I use force spectroscopy to determine the elasticity and structural properties of cells. Changes in these features are often associated with functional or pathological changes in the cell.

An important element of the presented analysis is the interpretation of experimental data. The two most commonly used models of cell elasticity were introduced by Hertz and Sneddon. They are single-parameter models that meet strong assumptions, including homogeneity of the material, small deformations, specific probe geometry, etc. Biological systems with a complex structure, such as endothelial cells, are far from homogeneous. Analysis of the measurements of force-distance curves reveals changes in local elasticity with the depth of indentation. Consequently, multi-parameter models are essential for correct data analysis.

The proposed solution to the problem is to use the layered material model, originally proposed by Kovalev to describe the elasticity of thin layers. This model was modified to accommodate the data collected for endothelial cells. The elasticity parameter obtained as a result of the analysis is described by a step function with transition zones in which there is an elasticity gradient.

The second research topic was the quantitative description of the surface structure of the membrane of the liver sinusoidal endothelial cells. For this purpose, the use of machine learning-based algorithms has proved to be a particularly useful method. As a result of this approach, an effective analysis of the structures in the topographic images from AFM was carried out.

It turns out that the accuracy of the proposed method not only exceeds the previous manual analysis, but also it is much faster, insensitive to image contrast and fully deterministic, therefore the results are more reliable. In addition, the presented scheme can be easily modified to classify different objects of interest, which favours the use of neural networks as a universal tool for the analysis of many types of microscopic images.

## Acknowledgments

The results described in this doctoral dissertation were achieved thanks to research carried out at the Faculty of Physics, Astronomy and Applied Computer Science of the Jagiellonian University, with the cooperation of scientists from the Jagiellonian Centre for Experimental Therapeutics and the Institute of Nuclear Physics, Polish Academy of Science. I would like to express my gratitude to these institutions and their staff.

A portion of this work was part of the research project SYMFONIA 3 supported by the Polish National Science Centre under grant agreement no.: UMO-2015/16/W/NZ4/00070, led by Prof. dr hab. Stefan Chłopicki. I also acknowledge the support of the Strategic Programme Excellence Initiative at Jagiellonian University, and the support from Jagiellonian Interdisciplinary PhD Programme.

I would like to thank my supervisors Prof. dr hab. Jerzy Konior and Dr Bartłomiej Zapotoczny. Their guidance enabled me to improve my ability to conduct research, as well as present and publish the results. I am also grateful to Prof. dr hab. Marek Szymoński and Prof. dr hab. Zbigniew Postawa, who were always helpful in solving any problems and often gave me scientific and administrative advice.

The appreciation goes to my colleagues and co-workers. First and foremost to Dr hab. Marta Targosz-Korecka, inż. Dr hab. Ewelina Lipiec, Dr hab. inż. Jakub Barbasz, Dr Leszek Krzemień, Dr inż. Izabela Czyżyńska-Cichoń, Dr inż. Kamila Sofińska, Dr Natalia Wilkosz, Dr Michał Kański and Mgr Katarzyna Małek-Ziętek. They allowed me to learn many necessary research skills from them.

Finally, I am thankful to my friends Gosia, Ania, Marysia, Olga, Kuba, Picek, Staszek and many others. I have always been able to count on your friendship and help, which have been invaluable to me. I also express my gratitude to my parents and the rest of the family, for their support and love.

At the very end, I wholeheartedly thank my husband, Krzysiek, for his encouragement and unending smile. They kept me going. And lastly, an honourable mention goes to our cat, Abi, for patiently sitting on my lap for the countless hours, while I was working on my PhD.

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# 1. Structure of the dissertation

This doctoral dissertation contains a description of selected methods and the results I obtained during my PhD research. In my studies, I concentrated mainly on the data analysis and interpretation of the outcomes of atomic force microscopy experiments carried out on endothelial cells.

The work is organized as follows:

In Chapter 2, I introduce the essential notions of atomic force microscopy and present the most imperative insights into endothelial cells in the context of my work.

In Chapter 3, I describe the motivation of my work, the scientific background and the expected results.

In Chapter 4, I present the outline of the analysis and findings. This chapter is divided into two sections. The first covers topics related to the mechanical properties of endothelial cells, and the second concentrates on the aspects of their morphology.

Chapter 5 is a recapitulation of my work. The most significant findings were collected at this place. I also lean over some questions that I was unable to answer and topics I did not cover in detail in this work, as I believe that they were an important part of my journey.

The dissertation summarizes and complements my results published in international peer-reviewed journals. However, it does not reproduce all the content presented there. The set of three articles, in which I was both first and corresponding author, are reprinted at the end of this work.

The list of publications, included as first three positions in the bibliography:

- 1) Magdalena Rusaczek (Giergiel), Bartłomiej Zapotoczny, Marek Szymonski, Jerzy Konior, *Application of a layered model for determination of the elasticity of biological systems*, Micron 124, 102705 (2019), DOI: 10.1016/j.micron.2019.102705 [1]
- 2) Magdalena Giergiel, Katarzyna Ewa Malek-Zietek, Jerzy Konior, Marta Targosz-Korecka, *Endothelial glycocalyx detection and characterization by means of atomic force spectroscopy: Comparison of various data analysis approaches*, Micron 151, 103153 (2021) DOI: 10.1016/j.micron.2021.103153 [2]
- 3) Magdalena Giergiel, Bartłomiej Zapotoczny, Izabela Czyzyska-Cichon, Jerzy Konior, Marek Szymonski, *AFM image analysis of porous structures by means of neural networks*, Biomedical Signal Processing and Control Volume 71, 103097 (2022), DOI: 10.1016/j.bspc.2021.103097 [3]

## 2. Introduction

### 1.1. Atomic Force Microscopy

Atomic Force Microscopy (AFM) has been a ground-breaking tool in various areas of research since its invention by Gerd Binnig, Clavin F. Quate, and Christoph Gerber [4]. It is commonly used in applications of material science and nanotechnology [5], surface chemistry [6], molecular engineering [7], polymer science [8], physics [9], semiconductor science, and technology [10], as well as biochemistry [11], biophysics [12, 13], molecular biology [14], microbiology [15], cell biology [16], and medicine [17].

The basic features of AFM, its configuration, and the modifications applied to cell measurement were already widely discussed in the literature [4, 18, 19]. Therefore, in the next few paragraphs I only briefly mention the aspects closely related to my studies. I will shortly discuss imaging modes, AFM probes, calibration, and the influence of measurement artefacts.

Three main purposes of AFM are force measurement, topographic imaging, and manipulation. In my research I used the first two functions. To measure mechanical properties of endothelial cells, such as stiffness and effective Young modulus, I analysed force-indentation curves collected in force mapping mode [1, 2]. On the other hand, for high-resolution surface topography measurement, a new advanced mode called Quantitative Imaging (QI mode developed for NanoWizard 3 AFM, JPK) was used [3].

Force-indentation curves measured in force spectroscopy mode may contain information about vast variety of interactions, including not only elasticity [20-22], but also adhesion [23-25], van der Waals forces [26, 27], electrostatic forces [28, 29], magnetic forces [30], friction [31], and lateral forces [32, 33]. Such a potential wide range of interactions make the interpretation of force-indentation curves immensely difficult. It is crucial to limit the impact of forces that are not under study as much as possible and to choose an appropriate model to interpret the data. In this work, the elasticity is of greatest interest. The discussion of commonly used elasticity models is provided in Chapter 4.

As mentioned before, the data for the evaluation of mechanical properties and topography were acquired in two different modes: Force Volume (FV) and Quantitative Imaging (QI). In fact, they are both contact modes, but they differ in a few details that optimize the measurement. The FV mode is characterized by a smaller maximal frequency, a longer acquisition time, and a smaller image resolution compared to QI mode [34]. The tip path between contact points with the sample is optimised in QI mode to enable a faster scan. The acquisition frequency influences the shape of the force-indentation curve and the noise level [35, 36], and therefore the final results of the elasticity. Depending on the tip size and shape, the maximal frequency of data acquisition, for which elastic models remain relevant, should be determined in the experiment. Above that threshold, there is an increase in the Young modulus versus frequency [34, 36].

Another factor influencing the data analysis and the final results of the elasticity measurements is the tip geometry [37, 38]. The measurements with blunt probes (the radius of curvature above 200 nm), or spherical (colloidal) probes usually give better results due to geometry which is easier to characterize and finer fitting to elasticity models.

A further important point is the calibration of the cantilever spring constant [39]. The manufacturers of AFM equipment suggest three methods. First is the direct calculation of the spring constant from the cantilever geometry. Unfortunately, this method is not very reliable due to strong dependence on the cantilever dimensions and possible variations in cantilever shape.

The second method is the measurement using a reference cantilever. This approach also has many disadvantages. Among others, it requires having a very well calibrated reference cantilever and performing a complicated calibration procedure [40]. The third method, which uses thermal noise measurement, is the most appropriate in our situation. The procedure is simple and may be performed in liquid in which the experiments are performed [41].

In the context of calibration and initial preparations for the experiment, it is necessary to mention a large collection of research aimed at standardizing nanomechanical AFM procedures (SNAP) for measuring biological samples [38]. It was carried out in 11 independent laboratories, on various instruments, and by different operators. The investigation concentrated on the proper determination of deflection sensitivity, based on the value of the cantilever spring constant measured with vibrometer. The research shows that establishing the calibration from the thermal fluctuations is error-prone. Unfortunately, mere knowledge of the correct value of the spring constant is not enough. It is necessary to compute the correction factor for the deflection sensitivity, using the force constant from the thermal noise and the independently measured value. Although this approach gives excellent results, it requires purchasing expensive, precalibrated cantilevers or having a vibrometer in the laboratory.

Finally, after a brief summary of the most important issues related to the AFM experiments, I finish this section with a description of selected advantages and disadvantages of AFM. First of all, unlike scanning electron microscope (SEM), AFM does not require any damaging sample preparation, such as metal coating, etc. It is possible to observe endothelial cells both fixed [42, 43] and living [44, 45] in their native environment, that is, in liquid. The measurement can be conducted with high resolution, enabling detailed surface analysis. Lastly, AFM gives the ability of measuring a three-dimensional surface profile, providing simultaneously information about the topography and stiffness of the sample.

However, coupled with these undeniable benefits, there are some disadvantages which must be taken into consideration. The image area for AFM is much smaller in comparison to that for SEM. In addition, in AFM the height measuring capacity is limited. Samples with large height fluctuations are rarely suitable for AFM measurements without proper corrections [46]. Another problem is the speed of the measurement. Although there is considerable progress in high-speed AFM [47], the measurements are in general time-consuming, and may require thermal drift corrections [48]. Furthermore, the ill-considered and inappropriate choice of the probe can introduce artefacts, such as flattening real morphological features or inaccurate representing steep wells or overhangs [49]. In the context of elasticity studies, the techniques based on optical traps may give lower force noise and clearer contact point determination, however, they focus on lower force regimes [50].

## 1.2. Endothelial cells

Endothelial cells (ECs) are the main focus of my research. Current knowledge of the endothelium is very broad, so I mention only the key aspects of their morphology and mechanical properties. The endothelium is a highly specialized layer of epithelial cells that covers the internal surface of the blood and lymph vessels and the cavities of the heart. Its role is to form a protective, non-adherent layer and participate in the transportation of many substances between cells and the bloodstream. It plays a key role in permeability, leukocyte trafficking, and regulation of vascular tone [51, 52]. Vascular ECs are physiologically active and perform numerous essential functions in the body, such as regulating haemostasis, maintaining blood fluidity, and participating in coagulation cascade and formation of blood clots. They secrete a number of biologically active substances that stimulate platelet aggregation (factor VIII), inhibit blood coagulation (thrombomodulin), dilate vessels and inhibit platelet aggregation (nitric oxide and prostacyclin).

Endothelial dysfunction is a distinctive feature of many diseases. For example, long-lasting hyperglycemia causes stiffening of EA.hy926 endothelial cells, due to changes in the actin cytoskeleton [53]. Even after the cell line is returned to the normal glucose conditions, the cell stiffness stays at the elevated values. Another example is the association of endothelial dysfunction with atherosclerosis and its complications [54, 55]. In general, endothelial dysfunction is characterised by defective NO-induced vasodilation. It is also associated with traditional cardiovascular risk factors, such as hypertension, dyslipidemia, and metabolic syndrome.

Measurement of the mechanical properties of cells can be used as a marker of pathological changes, such as cardiovascular disorders [56] or cancers [57]. For instance, diabetic cardiomyopathy is related to abnormal cardiac contractile function and alterations in the mechanical properties of cardiomyocytes [58]. This and many other examples show that investigating the mechanical features of cells is crucial for the early detection and development of treatments of related diseases.

What is worth mentioning, nowadays the research on ECs is conducted both on isolated cells and continuously cultured lines. However, the results of measured mechanical properties may differ due to differences in culture conditions or changes occurring during the cell isolation process. Therefore, the results should be confirmed using *in vivo* and *in vitro* methods.

A part of ECs, whose abnormality may be related to vascular dysfunction is endothelial glycocalyx (eGlx) [59, 60]. It is a layer of glycolipids and glycoproteins that protects the cell surface against mechanical and chemical damage [61]. Importantly, the glycocalyx of different cell types differs in thickness and in the composition of saccharide residues. The characteristic configuration of the residues is responsible for the mutual recognition of the cells. eGlx is also responsible for regulating vascular tension and intercellular adhesion.

Another important feature of endothelial cells is their heterogeneity. In addition to different glycocalyx compositions, ECs have different cellular shapes, various types of intercellular junctions, etc. The shape of the EC also varies depending on the external conditions. In the native environment, the endothelium is exposed to fluid shear stress. Due to this interaction, it

undergoes a change in cell shape combined with microfilament network remodeling in the direction of flow [62].

Depending on the type of EC, they also differ in the elasticity parameter [63]. This feature is particularly important because due to the location inside the blood vessels, they are frequently stretched and squeezed due to the blood flow. Heterogeneity and complex structure require the use of appropriate models to interpret the nanomechanical properties of ECs [42].

According to Bennett [64], next aspect of EC heterogeneity is structural heterogeneity. The first measurement, conducted with the help of electron microscopy, enabled the classification of EC according to their continuity. The following cases can be distinguished [65]:

1. Cells without fenestrations or pores – continuous endothelium. Cells are tightly connected and anchored in a continuous basal membrane. This type is most common, can be found in the arteries and veins of the lungs, heart, and muscles.
2. Cells with intracellular fenestrations – fenestrated endothelium. This type is also attached to a continuous basal membrane, but has transcellular 50–60 nm wide pores, which are sealed by a thin diaphragm. These cells occur in places with an elevated filtration role, such as the endocrine and exocrine glands, the gastrointestinal tract, and kidney glomeruli.
3. Cells with intercellular fenestrations – discontinuous endothelium. Cells are attached to a crudely structured basal membrane and have larger pores (100-200 nm) without diaphragm. This type is present mainly in sinusoidal vascular beds in the liver and also in the spleen and bone marrow.

A decrease in the frequency of occurrence or size of fenestration (membrane porosity) may be a marker of pathological conditions in the liver [66]. Among them are primary liver diseases (cirrhosis, fibrosis, hepatitis), influence of liver toxins (oxidants, bacterial toxins), systematic diseases (diabetes), and other processes (aging, high pressure) [67]. Thus, examination of changes in ECs morphology gives insight into the condition of the liver. So far, a basic methodology for examining the structure of fenestration has been developed, but there has been no quick and automatic method for analysing microscopic images [68-71].

### 3. Motivation

Despite numerous studies on the subject of endothelial cells, their role, physiology, and our ability to restore them to normal functioning (in the event of dysfunction) still leave many questions. Especially, the evaluation in a liquid environment is quite new. The recent construction of devices of nanometre resolution enabled quantitative measurements. In addition, progressive automation and acceleration of measurement speed allow for a valuable quantitative analysis. However, the increase in measuring efficiency must be accompanied by the acceleration of the process of data selection and analysis.

In my research, the objective was to achieve two main goals.

The first was to find and test a relatively simple model that would be applicable to measure cell elasticity with AFM. The model should take into account the layered structure and the complexity of the biological systems under study. Furthermore, I wanted to include the local character of elasticity. That means that the elasticity parameter is a property defined in each small part of the sample, not a global property of the system. Very few force-indentation curve analyses in biology consider a differential approach that enables the precise determination of sample elasticity at various indentation depths. For a further discussion of the double layer model that was the result of my research, is in the next chapter.

To complete this part of my work, I also analysed the measurements that aimed to detect and characterise eGlx using the atomic force spectroscopy technique. Previous work on this topic made a great contribution to understanding cell physiology [72, 73]. However, their approach to data analysis was not compared to other possible analyses and the correctness of the assumptions was not confirmed. Therefore, a broader overview of possible methods of analysis was expedient to complete the research.

The second goal of my work was to develop a method for analyzing high-resolution AFM images, which allows a quick and effective classification of their elements. This task was carried out in the context of determining the surface porosity of liver sinusoidal endothelial cells. The possibility of imaging large areas of living and fixed cells with high special precision has opened the door to the study of quantitative changes in the amount of fenestration. However, automated analysis methods, which would allow for very objective and reproducible measurement results, have not been developed so far. The main idea in solving this problem was to use a convolutional neural network capable of classifying the elements of the image. Details of this approach are described in the further part of the work.

## 4. Methods and results

### 4.1. Theoretical models of elasticity

The cornerstone of most of the treatise on cell elasticity measurements using AFM is the Hertz model. It is based on the ground-breaking Hertzian contact theory [74], which was later applied by Harding and Sneddon to the indentation of the surface plane by a rigid conical punch, a rigid sphere, and a flat-ended cylindrical punch [75]. The model was thoroughly discussed in the literature, also in the context of biological materials [45, 76-78]. The basic assumptions are that the sample is isotropic, homogenous and linearly elastic, the contact is adhesionless and frictionless (there exist extended theories in these cases), and the indentation depth is small, as theory omits the influence of the substrate, there is also a condition limiting the indentation depth, which depend on the probe diameter.

The relation between force  $F$  and indentation  $\delta$  in the classical model is [75]:

$$F(\delta) = \frac{4}{3(1-\nu^2)} E \sqrt{R} \delta^{3/2} \quad (1)$$

Where  $E$  is the Young's modulus of the cell,  $\nu$  is the Poisson ratio of the cell (usually set to 0.5) and  $R$  is the radius of the probe. A common modification is a correction for the spherical shape of the cell ( $R_{cell}$ ) in the form of replacing the radius  $R$  by the effective radius  $R^*$  taking into account the shape of the cell:

$$\frac{1}{R^*} = \frac{1}{R_{cell}} + \frac{1}{R_{probe}} \quad (2)$$

Starting from this simple model, there are many ways to introduce the layered structure of the biological system. The course of action depends on the physical characteristics of each layer. In the case of two linearly elastic layers, e.g. the cortical layer and the nucleus, the simplest idea is to fit two independent Hertz-like functions to regimes of successive layers. This concept, although easy to apply, has some disadvantages. Most importantly, it is necessary to make a strong assumption in which ranges of indentation depth there are different parts of the cell. Of course, the difference in the parameter of elasticity between the individual fragments must be measurable.

The situation gets even more complicated, when we are not sure about the linear nature of the elasticity of any of the layers. For example, in the glycocalyx and the cell body system, the former may not be strictly linear.

Many previously proposed solutions are based on finite element method (FEM) modelling [63, 79, 80]. Such approaches, however, have the serious disadvantage that the obtained results (for example, in the form of proposed analytical formulas) are often not universal, and even a small change in geometry may completely change the model.

A key step in deriving an analytical solution that works under the assumption of a gradual change in elasticity throughout the sample was to look at the derivative of the force versus indentation curve.

$$\frac{dF}{d\delta} = 2\sqrt{R\delta} E(\delta) \quad (3)$$

As for the shape of the  $E(\delta)$  function, we must make some assumptions. Kovalev [81] proposed a very simple step function that takes values between the boundary values of the two layers  $E_0$  and  $E_1$ . Down to potential numerical problems, the idea of the double-layer model is simple.

First, from the experimental data, we calculate the derivative of the force-indentation curve. Then, from Equation (3), the dependence of the elasticity parameter on the indentation depth is determined. In the next step, we adjust to the experimental  $E(\delta)$  the best possible fit of the simplified step function (representing the change in elasticity with indentation depth). In the last step, we integrate the best fit of the model  $E(\delta)$  function to recover the force-indentation relationship in our model. To be completely accurate, the last step is not vital, since all the necessary parameters have already been calculated. However, it is worth doing this part to confirm the quality of the fit. An extensive description of all technical details is in [1].

The layer model realized in this way was implemented for the data of the LSECs elasticity measurements [1]. It was also compared with other models of elasticity, based on the measurements of the elasticity of endothelial cells covered with glycocalyx [2].

The brief summary of the results:

Double-layer model showed a difference in the elasticity of ECs from mice fed a high-fat diet compared to the standard diet [1]. This effect was not noticeable with the single parameter model. A more detailed analysis indicated that changing the diet did not affect the cortex layer, while for the deeper layers of the cell (which correspond to the nucleus), cells from mice on a high-fat diet are stiffer.

In the second work, the double-layer model was applied to ECs covered with glycocalyx layer, that was enzymatically removed and later restored [2]. The model was directly compared to the simple contact mechanics model based on stiffness tomography method [82]. The double-layer model led to similar results, but was more flexible in adjusting the searched parameters of the sample. In particular, it did not need to assume the thickness of the glycocalyx, but determined it based on local elasticity.

In the end, I must emphasize that all models are only idealized approximations of a real physical system. There is no gold standard for measuring the values of ‘real’ elastic parameters. Nevertheless, the nanoindentation method with the AFM probe combined with a suitable model is useful for monitoring changes in samples due to various processes.

## 4.2. Application of neural networks to the study of ECs morphology

The use of machine learning and neural network-based algorithms is becoming more and more popular in the analysis of microscopic and spectroscopic data [83-85]. The assumptions that were made during the formulation of the research problem (creating a fast, objective, and precise method of classifying the elements in the AFM image) ideally justify the use of convolutional neural network methods.

The input data are in fact a set of force-indentation curves collected at successive points of the measured area. From these curves, it is possible to deduce a number of features that characterize each point on the created image. If we determine the point of contact at each point of the created "map", or the position of the probe at for constant force of indentation, we will obtain an image of the sample topography (height). On the other hand, when we measure the rate of change in the slope of the force-induction curve, we get an image of the stiffness of the sample.

The application of neural networks made it possible to teach the algorithm how to recognize features that have image fragments with fenestrations. What is important, we can use the height and the stiffness of the sample at the same time. The whole process of creating, testing, and using the method is described in detail in [3]. In summary, the most essential steps are:

1. Normalization – recalculation of the physical values of height and stiffness to a scale from 0 to 1 for correct operation of algorithms that take values only in this range. I use the linear transformation.
2. Creating a training data set – there are two main reasons for this step. First, to increase the amount of data used to train the neural network and second, to remove unwanted biases. The elements of the training set are created by adding to the existing images additional noise that occurs naturally in the experiment. Additionally, one can apply geometric transformations such as rotations and reflections.
3. Finding a proper architecture of the network and loss function – the network function transforms each image pixel into a vector of classes, with values describing likelihood of a given feature being present there. Loss function returns real numbers – loss, which is minimal, when the network output is the same as class present in the pixel in the training set. The loss function together with the network transforms images to real numbers, which can then be minimized, so the accuracy of the classification increases. The network is composed of layers of functions, each taking the results from previous layer until the final output layer. The type of connections used in the article was based on previous works in image classification, modified to account for the fact that the features we look for have just a few or even a single pixel areas. Different loss functions were tested and all seem to give similarly accurate results pointing to the robustness of the machine learning approach.

The algorithm created and tested in this way was used to calculate the porosity of images with different incidences of fenestration. The most important conclusion is that the calculated number of fenestrations correlates strongly with the expected value based on the manual count. Therefore, the goal set at the beginning has been achieved. The presented method has already been used several times during cooperation with scientists from other research units.

The brief summary of the results:

The method based on neural networks leads to a comprehensive analysis of spectroscopic data [3]. Starting with raw data, it is possible to quickly determine the frequency of fenestration occurrence, regardless of the noise and measurement artefacts involved. The presented approach works for different porosities of membrane in the image and with different pore sizes. Moreover, an additional advantage is the possibility of a rough estimation of the size of fenestration, even with a relatively low image resolution. For a more accurate determination of the size of the pores, of course, measurements are needed at a higher resolution. However, larger resolution does not allow for the imaging large fragments of the membrane in a short time.

## 5. Summary

### 5.1. Main results

In this work, I presented the problem of quantifying the mechanical and structural properties of endothelial cells. It was a recapitulation of a collection of three articles [1-3]. The body of work presented in abovementioned papers is the main part of my PhD scientific achievements. The work is characterized by a broad approach to the study of ECs using AFM, presenting many research techniques and an extensive analysis that surpasses the approaches presented so far. It is worth noting that different types of endothelium are investigated - the continuous ones, from cell lines and the discontinuous ones with fenestration, which must be primary, because there is no line that would preserve the fenestration morphology.

For the conclusion, first, I discussed the issue of determining the elasticity parameter using atomic force spectroscopy. This topic arouses a wide discussion among physicists and scientists who use this method for biological measurements [86]. The main problem is the strong dependence of the determined elasticity parameter on the indentation depth, which should not be the case if the conditions for the applicability of the popular Hertz and Sneddon models are met. The solution I use is the adaptation of the double-layer model, which takes into account the gradual change of the Young's modulus along with the depth of indentation.

Second, a proprietary method of high-resolution AFM images analysis was presented, using both the topography and the local stiffness of the measured samples. The use of the convolutional neural network allowed for quick and effective classification of individual elements of the image. Concurrently, the analysis is less time-consuming and more objective. An additional advantage is the universality of the created algorithm, thanks to which it can be used for other types of microscopy, after appropriate modifications.

My work consisted mainly of programming, theoretical modelling, data segregation, statistical analysis, and similar activities. For modelling and data analysis, I used a number of different tools, among them Wolfram Research Mathematica, Matlab, AtomicJ [87], ImageJ [88], etc. as well as my own code written in Python and C++.

It is worth noting, however, that I also acquired a number of practical, experimental skills, such as operating AFM in several modes and unsupervised handling of all parts of the measuring devices (such as the liquid cell with temperature control or the optical system needed for AFM tip calibration). Furthermore, I learned how to conduct a simple cell culture, work in a fume hood, etc. My work involved understanding the principles of operation of all measuring devices, as well as the physical properties of the samples, such as elasticity, adhesion, friction, electrostatic interactions, etc.

The interdisciplinary nature of my studies was rewarded with the opportunity to participate in the Jagiellonian Interdisciplinary PhD program, thanks to which I could expand my research workshop, increase my scientific and professional potential, and learn about research practices in other areas and disciplines of science. Furthermore, participation in the program gave me the opportunity to attend international scientific workshops and conferences.

## 5.2. Other collaborations and research

The preceding part of this report is not a complete picture of my PhD research pursuits. Therefore, here I briefly outline a few more projects and research that shaped my knowledge on the topics related to my PhD.

I started my research trying to find a more complex layer model that fits a tissue sample. In retrospect, this task had little chance of success, especially for an inexperienced researcher. The analysis of such a complicated, uneven system, which is additionally susceptible to damage during preparation and thus measurement disturbances, is extremely difficult. In such complex cases, the analysis requires surgical precision, similar to that of a scalper operator who prepares the sample for measurement (see, for example [89]).

After this experience, I started to analyse single cells, which are characterised by a more homogeneous geometry and mechanical properties compared to tissues. My sandbox was a project on cardiac microvascular endothelial cells (CMECs). In this project I had the opportunity to learn the principles of atomic force spectroscopy, basic data analysis, and how to pay attention to and avoid potential errors. In this project, I learned a lot from Prof. Marta Targosz-Korecka.

Later on, I adapted for biological purposes a double-layer model based on the work of Kovalev and applied it to LSECs. At that moment, I was curious to know what class of data this model can be used for. I had the great opportunity to apply the model to human aortal endothelial cells (HAECs). In contrast to LSECs, these cells have a fairly rich layer of glycocalyx on the membrane surface. Nevertheless, the model fit reasonably well, despite quite different basic assumptions.

I found the idea of a model that combines layers with different types of elastic properties very compelling. I investigated the model presented by Delgadillo et al. [80], and previously described by Clifford and Seah [90]. The authors developed an analytical formula for the case of a compliant layer on a stiffer substrate. It was based on the results of finite element modelling. Unfortunately, despite many efforts, I was unable to apply this model to our experimental data. I tried both my own code and the method provided by the authors. Eventually, I figured out the reason of that trouble. The Kovalev's model has 4 fit parameters: two elasticities, the thickness of the first layer and a parameter that can be interpreted as the rate of transition between layers, while the Clifford's model has only 3 parameters. The rate of transition is determined by the numeric constants in the equations. Fitting this model to other glycocalyx characteristics would probably require modification of the formula or repetition of the finite element calculations.

At that moment, I tried to get a glimpse of finite element analysis (FEA). I was lucky to get the opportunity to join a project conducted by the group of Prof. Jakub Barbasz from the Jerzy Haber Institute of Catalysis and Surface Chemistry of the Polish Academy of Sciences. Thanks to established cooperation, I learned the basics of FEA while working on a project on the role of cortex in the AFM indentation experiments. The results of this work were presented during 33rd M. Smoluchowski Symposium on Statistical Physics and are outlined in the article titled *"The role of the cortex in indentation experiments of animal cells"*, which at the time of writing this doctoral dissertation is under review for a scientific journal. The main conclusions resulting from this investigation are the following: while treating the cell cortex and the inner parts of

the cell as linearly elastic materials, we were able to suggest an analytical expression including the influence of the cortex in the Hertz model. In addition, we discussed various experimental factors such as the effect of cell size and shape, off-center indentation, and the effect of friction.

In the meantime, I came across an article exploring the properties of glycocalyx with a completely different method – using a coarse-grained approximation of molecular dynamics (MD) [91]. After a crash course of MD in the group of Prof. Zbigniew Postawa, I was able to reproduce a large part of the results from the previously mentioned article and create similar models. The results were very informative and gave me a new image of the mechanical properties of the glycocalyx. After that I was able to give a two-part seminar titled “*Application of molecular dynamics to glycocalyx modelling*” for my Department of Physics of Nanostructures and Nanotechnology. During my presentation, I compared the MD approach to the brush model presented by Sokolov. Some of the conclusions were that the linear relationship between force and probe size is correct up to the indentation to about half of the glycocalyx length and that the glycocalyx response is weakly dependent on the rate of indentation. Both MD and FEA modelling gave me an educational experience of conducting relatively large numerical computations.

On the other hand, in addition to studies on the endothelium, I also had the opportunity to participate in studies on other cell types. Together with Prof. Marek Szymoński and with a tremendous help of Prof. Ewelina Lipiec and Dr. Kamila Sofińska, we conducted preliminary studies of the influence of substrate roughness on elasticity of the osteoblastic cell line MG-63. The research was carried out with the participation of Prof. Andrzej Kotarba group from the Faculty of Chemistry, Jagiellonian University. The objective was to investigate the influence of parylene C substrates on osteoblast elasticity in the context of potential applications in implantology [92-94]. Unfortunately, after a promising start, this project was quite abruptly interrupted by COVID-19 lockdowns and restrictions on laboratory work.

As part of my doctoral internship, I visited the Max Planck Institute for the Science of Light in Erlangen, Germany. During the internship, I measured the mechanical properties of mouse isolated leukocytes with the use of AFM. I investigated the biophysical changes underlying the absence of the interleukin type 3 receptor (IL3) and also its absence in different mouse models (IL3KO and IL3RKO). This research was part of an ongoing scientific collaboration between the Max Planck Institute and the University Hospital in Erlangen. The research was of particular interest to me due to the different characteristics of the cells. In this case, they are relatively large, spherical, and nonadherent. During measurements, I used handmade wedged cantilevers [95]. Furthermore, the analysis used a double contact model taking into account the cell deformation near the substrate [96].

Recently, after completing the research for my doctorate, I continued cooperation with scientists from Jagiellonian Centre for Experimental Therapeutics and the Institute of Nuclear Physics, Polish Academy of Science, to develop methods of pattern recognition in the images of endothelial cells, with the intention of generalizing the method for use in other techniques such as scanning electron microscopy (SEM), and structured illumination microscopy (SIM).

I am also part of a thriving group of Prof. Ewelina Lipiec, with the aim to learn data analysis from other techniques, complementary to AFM. I deal with spectra from the Raman spectroscopy measurements (principal component analysis) and the fluorescence images from the confocal microscopy.

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## Additional achievements

### Scholarships and projects

- 2019 – 2021 PhD student in the Jagiellonian Interdisciplinary PhD programme
- 2019/2020 Jagiellonian University Pro-quality Scholarship
- 2016/2017, 2021/2022 Jagiellonian University Rector's Scholarship for PhD Candidates
- 2022 PhD student in National Science Centre OPUS 19 Grant „*Direct monitoring of the dynamics of local changes in the secondary structure of peptides in Alzheimer's disease during aggregation*”
- 2021 – 2022 Principal investigator of Priority Research Areas of the Jagiellonian University SciMat Grant „*Application of machine learning to pattern recognition in atomic force microscopy images*”
- 2021 Principal investigator of Priority Research Areas of the Jagiellonian University DigiWorld Grant „*Analysis of microscopic images of biological nanostructures using neural networks*”
- 2019 – 2020 Principal investigator of Jagiellonian University Grant „*Study of the endothelium nanomechanics of cardiac microvessels by means of atomic force spectroscopy*”
- 2018 – 2020 PhD student in National Science Centre SYMFONIA 3 Grant „*Pharmacotherapeutic mechanisms, stereoscopic signatures and nanomechanics of dysfunctional endothelium in liver steatosis and heart failure; in search of organ specificity*”
- 2015 – 2018 Teaching assistant: Fundamentals of Physics: Thermodynamics  
Teaching assistant: Physics Lab I  
Substitution for courses in Mechanics and Mathematics

## Conferences, internships and schools

- 30 V - 5 VI 2022  
Le Gosier, Guadeloupe      3rd NANOinBIO Conference, **talk** „Automation of data processing for AFM image classification” and Spring School „Machine Learning for Data Analysis”
- 24-28 VII 2021  
Vienna, Austria      13th European Biophysics Conference, **poster** „Machine learning approach to pattern recognition in atomic force microscopy images”
- 7-15 VII 2021  
Virtual Budapest, Hungary      Eastern European Machine Learning Summer School 2021
- 28 VI - 23 VII 2021  
Erlangen, Germany      Internship at Max Planck Institute „Investigation of the biophysical changes underlying the absence of the interleukin type 3 receptor (IL3) in different mouse models (IL3KO and IL3RKO)”
- 8 VI 2021  
Erlangen, Germany      Seminar **talk** at Max Planck Institute „Mechanical and structural properties of endothelial cells”
- 3-4 XII 2020  
Zakopane, Poland      33rd M. Smoluchowski Symposium on Statistical Physics, **poster** "Finite element modelling of atomic force indentation of an animal cell"
- 20-24 VII 2020  
Marseille, France      12th AFMBioMed Summer School
- 31 I - 3 II 2020  
Linz, Austria      XXII. Annual Linz Winter Workshop 2020, **talk** „AFM image analysis of porous structures by means of neural networks”
- 21-25 X 2019  
Grenoble, France      11th AFMBioMed Summer School
- 13-18 IX 2019  
Kraków, Poland      45. Congress of Polish Physicists, **poster** „Application of the layered model to determine the elasticity of biological systems”
- 28 XI-2 XII 2018,  
Zakopane, Poland      X Seminarium STM/AFM, **talk** „Application of the layered model to determine the elasticity of biological systems”
- 4-8 IX 2017,  
Kraków, Poland      8th AFM BioMed Conference, as participant and co-organizer
- 30 XI - 4 XII 2016,  
Zakopane, Poland      IX Seminarium STM/AFM, **talk**, „Nanomechanical properties of cells - construction of the theoretical model”
- 22-25 VI 2016,  
Poznań, Poland      NanoTech Poland 2016, **poster** „Theoretical interpretation of force spectroscopy measurements in the context of elastic properties of biological cells and tissues”

## Reprints of the publications

### Application of a layered model for determination of the elasticity of biological systems

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#### **Micron**

Volume 124, September 2019, 102705

<https://doi.org/10.1016/j.micron.2019.102705>

#### Author's contributions

Writing the script for analysis and testing it,  
Introducing the necessary changes to the model in order to adapt it to the tested ECs,  
Taking into account noise and boundary conditions,  
Conducting a complete data analysis using a two-layer model,  
Dominant contribution to the writing of the article, acting as a correspondent author.



# Application of a layered model for determination of the elasticity of biological systems

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## ARTICLE INFO

### Keywords:

Nanomechanics  
Cell elasticity  
AFM  
Liver sinusoidal endothelial cells  
Layer model

## ABSTRACT

Elasticity of biological systems is considered to be an important property that might be related to functional or pathological changes. Therefore, careful study and detailed understanding of cell and tissue elasticity is crucial for correct description of their functioning. Atomic Force Microscopy (AFM) is a powerful technique, which allows for determination of the physical properties, such as elasticity, of soft-matter systems in nano-scale. An important step in AFM elasticity studies is a proper interpretation of experimental data. Two most frequently used theoretical schemes applied to determine elasticity are due to Hertz and Sneddon, which are effectively one-parameter models. In this work, we go beyond this approach. Firstly, as elasticity is a local property, we extract from the slope of experimental force-indentation curve an elasticity parameter, which varies with indentation depth. Then secondly, we find best approximation of this parameter by applying the two-layer model with four effective parameters, as proposed by Kovalev. This method is employed to the experimental data taken on murine liver sinusoidal endothelial cells in non-alcoholic fatty liver disease model. The obtained results show additional effects, not seen within the traditional, simplified scheme. Namely, the elasticity of the first layer does not change its value in the model of non-alcoholic fatty liver disease, but the increase of stiffness is noticed in second layer. The second goal of this article is to reveal and discuss the differences between traditional approaches and the one being presented. The deviations from the original assumptions are analysed and the corresponding restrictions on utility of theoretical models are presented.

## 1. Introduction

Atomic Force Microscopy (AFM) was invented by Binnig et al. (1986) in 1986 and since then it has become a potent technique for determining physical properties of soft-matter systems in the nano-scale (A-Hassan et al., 1998; Radmacher et al., 1992; Vinckier and Semenza, 1998). The method is particularly useful, as by detecting forces between a probe and a sample, both sample topography and physical properties may be studied simultaneously. The main advantages of using AFM to examine biological systems are well established. Firstly, it allows one to study cells and tissues without damaging them. Secondly, it has excellent resolution, which grants the possibility to observe individual parts of the system. Lastly, it makes it possible to make the measurements in liquid environment, close to physiological conditions, with a gradual change of parameters, i.e., different pH, additional solutes, etc. (Hansma and Hoh, 1994; Radmacher, 2007; Kronlage et al., 2015; Zapotoczny et al., 2017).

AFM nanomechanical probing is widely used to study organic materials, starting with biomolecules, through isolated single living or

chemically fixed cells (Targosz-Korecka et al., 2015) ending with complex tissues (Graham et al., 2010). The validity of the method was discussed and reviewed in detail (Szymonski et al., 2015). It was found useful in diagnostics and medical research (Lekka et al., 1999; Liu and Webster, 2007; Fels et al., 2014).

Elasticity of biological systems is important property related to physiological processes. It was shown that the parameter is an early indicator of pathological state of cells (Dulinska et al., 2006). Moreover, it can be used to distinguish between cells (Lekka et al., 1999, 2012; Szczygiel et al., 2012).

In AFM studies of nanomechanical properties, a cell is probed by a sharp or colloidal tip and the relation between the loading force and the scanner position is recorded. These force-distance curves are then the subject of a theoretical analysis, which is a crucial element of the AFM elasticity studies. In the first step, the reduced force-indentation curves are generated, resulting from the subtraction of the calibration curve recorded on the stiff substrate from the curve recorded on the actual investigated samples (Weisenhorn et al., 1993).

For the analysis of the reduced force-indentation curves, two

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simplest and most frequently used theoretical models, taken from the classical theory of elasticity and used to determine elasticity characteristics, are due to [Hertz \(1882\)](#) for spherical indenters, and [Sneddon \(1965\)](#) for conical or paraboloidal ones. In both cases, given the geometry of the indenter, the loading force  $F(\delta)$  as a function of the indentation  $\delta$  may be calculated, provided the material elastic properties, i.e., Young's modulus  $E$  and Poisson's ratio  $\nu$ , are known. For the actual experiment analysis,  $F(\delta)$  is taken from experiment, and then the parameters are extracted by fitting to the model function.

The aforementioned models were originally proposed to describe systems which satisfy some strong assumptions, including homogeneity of the material, isotropy, infinite thickness, small depth of indentation and deformations, specific probe geometry, etc. Unfortunately, in most cases, biological systems are heterogeneous, and often consist of layers. As a consequence, using those models is inevitably associated with making additional approximations, like using the effective Young moduli.

As biological systems have layered structure, it is possible to observe changes of elasticity with the increasing indentation depth ([Guz et al., 2014](#)). Typically for endothelial cells, the outermost layer of a cell is a glycoprotein coating, called glycocalyx, next there is a cell membrane supported by the actin and/or tubulin cytoskeleton, which surrounds cellular organelles including the cell nucleus.

It has been demonstrated ([Oberleithner et al., 2009](#); [Peters et al., 2012](#)) that a typical indentation curves of living endothelial cells in some cases do not present a simple power shape, rather, the indentation curves reveal at least two slopes. Moreover, the length of the first linear slope is highly variable from a few nanometers to several hundred nanometers. Deeper indentation leads to the appearance of a second linear slope. It is assumed that the first slope represents the plasma membrane stiffness and includes a contribution from the submembrane cortical cytoskeleton, whereas the second slope, which is approximately three times larger than the first slope, represents the stiffness of the inner bulk of the cell. In the described analysis, both layers are treated on the basis of the Hertz-Sneddon approach. The results directly suggest the multi-layer nature of the investigated materials, for which the Hertz/Sneddon model appears to be insufficient.

Different approach to multi-layered systems was presented by [Sokolov et al. \(2007, 2013\)](#) who studied molecular brushes on living cells. Authors introduced a method of analysis that decouples brush from the deformation of the cell body. While for the cell body deformation, the Hertz-Sneddon model is still utilised, the brush-probe  $F_{\text{brush}}$  force is described by the steric interaction known for polymeric brushes. Using AFM, [Iyer et al. \(2009\)](#) studied cell mechanics of the molecular brushes on living cells and they showed that normal and cancerous cervical cells yield different surface brush properties. Then, the brush model was used to explain differences in elastic properties between firmly and loosely attached cells ([Dokukin et al., 2013](#)). Recently, the brush model was effectively used to showcase the degradation of glycocalyx and stiffening of endothelium in mouse model of diabetes ([Targosz-Korecka et al., 2017](#)) where a complex analysis procedure revealed changes in both investigated layers. Another example of research using this model is the study of adhesion between pulmonary human aorta endothelial cells and lung cancer cells ([Malek-Zietek et al., 2017](#)) or the study of load rate and temperature dependent mechanical properties of the cortical neuron ([Simon et al., 2016](#)).

To include the effect of heterogeneity, another approach ([Park, 2017](#)) was introduced and that uses the asymptotic hyperbolic fit, in which the limiting behaviour of a layered structure is explicitly taken into account. Namely, for small indentation  $\delta$ , the elastic modulus  $E$  is this of the cell cortex  $E_C$ , while for large values of  $\delta$ , the elastic moduli approaches that of cell body,  $E \rightarrow E_B$ . In this approach, the investigated cell is considered to be built of two layers: a thin actin-rich cortex and a cytoplasmic layer with cytoskeletal components and a nucleus. After performing the analysis of the force-indentation curves, the elastic moduli of both layers were determined.

[Kovalev et al. \(2004\)](#) developed an approach for analysing AFM probing of very thin multi-layer polymer films on solid substrates. The model comprises two- or three- step graded functions with the transition regions that account for a gradient of the elastic parameters between the layers. So far, this approach has not been applied to elasticity studies of biological systems, i.e., cells or tissues.

Liver sinusoidal endothelial cells (LSECs) possess delicate structure which has hampered investigation of those cells in living state for decades ([Braet et al., 1998](#); [Braet and Wisse, 2012](#)). Recently, we demonstrated that the detailed morphology of live LSEC can be investigated with use of AFM ([Zapotoczny et al., 2017](#)). LSEC line hepatic sinusoids ensure very effective transport of solutes and macromolecules between the blood stream and interior of the liver ([Braet and Wisse, 2002](#)). Cell mean height is 2.2  $\mu\text{m}$  and is often below 2.0  $\mu\text{m}$  in the highest, nuclear area. Outside the area of the cell nucleus (which have the diameter of 10  $\mu\text{m}$ ), cell height rarely exceed 500 nm. Moreover, the peripheral parts of cells are filled with fenestrae, gathered in sieve plates, with height below 200 nm. It has been shown that many external factors, such as hormones or drugs, can alter the number of fenestrae ([Braet and Wisse, 2002](#); [Cogger et al., 2013](#); [Le Couteur et al., 2002](#)). It changes also in pathological state of the liver. Particularly, in non-alcoholic fatty liver disease, liver steatosis and fibrosis lead to loss of LSEC fenestrae ([Clark et al., 1988](#); [Deleve, 2016](#); [Maslak et al., 2015](#)). While fenestrae are built of actin ([Monkemoller et al., 2015](#); [Muller et al., 2016](#); [Oie et al., 2018](#)), which is also one of the main components of cell cortical layer elasticity, it would be particularly interesting to investigate changes in nanomechanics of LSEC in different conditions. However, only one report was found in the literature, where rat LSECs were investigated in both live and fixed state and elastic moduli were determined for a conical indenter ([Braet et al., 1998](#)).

In this report, we determine elastic moduli for murine primary LSECs. We present elasticity moduli of LSECs from mice on control and high fat diet. We compare results obtained using the classical Sneddon approach with the multi-layered analysis, indicating the necessity of application of such detailed approach for endothelial cells from liver sinusoids.

## 2. Methods

### 2.1. Cell isolation

Murine LSECs were isolated according to the original procedure, described by [Smedsrod and Pertoft \(1985\)](#) with previously described modifications ([Zapotoczny et al., 2017](#)). After isolation, LSECs were seeded on uncoated glass coverslips in EGM-2 medium (Lonza) at 37 °C in an atmosphere containing 5% CO<sub>2</sub>. LSECs were cultured for 15–24 h and then elasticity parameters were examined using AFM.

### 2.2. AFM

After 14 h of culture, glass coverslip was transferred from the culture dish to the BioCell™ (JPK Instruments). Measurements were conducted in fresh EGM-2 medium (Lonza) containing 2% fetal bovine serum (FBS) with addition of 20 mM HEPES buffer (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) at 37 °C under ambient conditions using a Nanowizard 3 (JPK Instruments) AFM system. Measurement of LSECs elasticity was conducted within the time of 3 h after seeding. Experiments were performed using Novascan V-shaped cantilever with a nominal spring constant 0.01 N/m with a colloidal polystyrene tip of diameter of 7.9  $\mu\text{m}$  with chemically non-modified surface. Measurement were conducted in force mapping mode.

Before measurements automatic spring constant calculation build in JPK Processing software were applied as a fit to thermal fluctuations of the cantilever. Then, the calibration curve was acquired on a glass surface for the same sample in the area without cells. The standard linear fit to the force distance curve was used to estimate cantilever

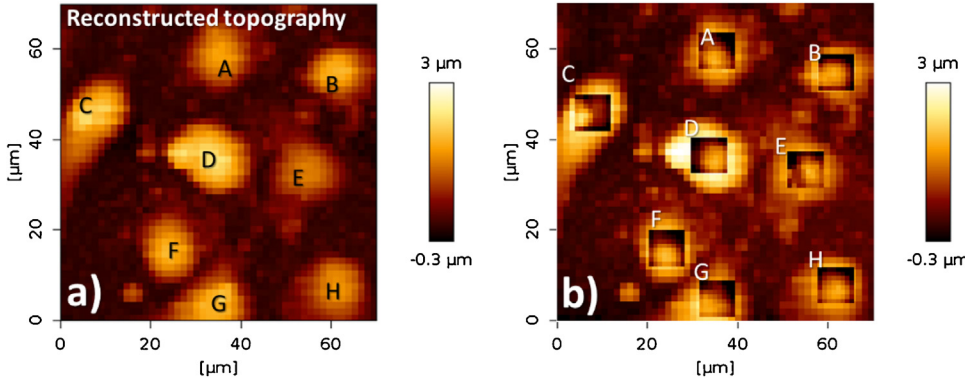


**Fig. 1.** LSECs measured using colloidal probe in force mapping mode. (a) presents the topography reconstructed for a loading force of 300 pN, where 8 cells were indicated with letters from A to H. (b) in the perinuclear zone  $8 \times 8 \mu\text{m}^2$  zones were selected in which additional force mapping with loading force of 1.0 nN was performed. Image size and resolution: large image  $70 \times 70 \mu\text{m}^2$ ,  $50 \times 50$  points, small image  $8 \times 8 \mu\text{m}^2$ ,  $8 \times 8$  points.

deflection and to control the force applied during the measurements.

Accounting for the calibration curve in detail was conducted in the later phase of data analysis and described in Section 3 and Supplementary materials, Section 1.

While LSEC nucleus has about  $10 \mu\text{m}$  in diameter it was difficult to precisely perform a map on the cell nuclei using an optical view only. Therefore, we divided AFM measurements into two parts. Firstly, a group of four to ten cells were chosen and a low-resolution map of force-distance curves was acquired, i.e., an image of  $70 \times 70 \mu\text{m}^2$  and  $50 \times 50$  force-distance curves were performed (low loading force of 300 pN were used) (Fig. 1a). Sometimes smaller or larger images were acquired, but the image resolution was calculated to remain constant. Such low-resolution maps allow us to reconstruct topography images of investigated area using JPK Data Processing Software and precisely choose the cell nuclei areas of investigated cells. Finally, we performed force maps in the cell nuclei areas (Fig. 1b). At the surface area of  $8 \times 8 \mu\text{m}^2$  always  $8 \times 8$  force-distance curves were acquired with acquisition speed of  $2 \mu\text{m/s}$  and the loading force of 1.0 nN. We further analyze those force distance curves, which corresponds to the flat regions over the cell nuclei.

### 2.3. Two-layer approach

In the approach developed by Kovalev et al. (2004), the starting point is the general relation between the surface stiffness ( $dF/d\delta$ ;  $F$  – force,  $\delta$  – indentation) and the local elastic modulus, proposed by Sneddon:

$$\frac{dF}{d\delta} = \frac{2\sqrt{A}}{\sqrt{\pi}} E', \quad (1)$$

where  $E'$  is the effective elastic (Young) modulus and  $A$  is the area of the tip-surface contact. The effective elastic modulus fulfills the following first-order differential equation:

$$\frac{dE'}{d\delta} = kE'(\delta), \quad (2)$$

with the coefficient  $k$  given by:

$$k = \alpha[(E_1 - E_0) - E'(\delta)], \quad (3)$$

while  $E_0$  and  $E_1$  are two values of the elasticity parameter for the components in the considered two-layered system;  $\alpha$  is a parameter measuring the width of the transition zone between the layers, and it may be either positive or negative, depending on the relative relation between the elastic properties. The value of  $\delta_0$  is the so-called reference depth, for which:

$$E'(\delta_0) = \frac{E_1 - E_0}{2}. \quad (4)$$

With the above assumptions, Eq. (1) can be integrated giving the following formula for the elastic modulus variation along the vertical coordinate  $\delta$ :

$$E(\delta) = E_0 + E'(\delta) = E_0 + \frac{E_1 - E_0}{1 + \exp[-\alpha(E_1 - E_0)(\delta - \delta_0)]}. \quad (5)$$

Eq. (5), describing microindentation in layered solids, was used by Kovalev et al. (2004) for analysis of the AFM results obtained for polymer films on solid substrates. In our case the thickness of the first layer is of the same order of magnitude, therefore the model is applicable. In Eq. (5), the four parameters  $E_0$  and  $E_1$ ,  $\delta_0$  and  $\alpha$  are to be determined from fitting the calculated curve to the experimental data.

For the Hertzian approach, the contact  $A$  can be estimated by  $A = \pi R\delta$ , where  $R$  is the radius of the indenter. Therefore Eq. (1) transforms to:

$$\frac{dF}{d\delta} = 2\sqrt{R\delta} E(\delta). \quad (6)$$

In the traditional approach, the elastic modulus is assumed as being constant and in such a case, Eq. (6) with simple integration yields the Hertz formula. However, we assume  $E(\delta)$  as a local function of the indentation, and Eqs. (5) and (6) represent the main ingredients of the fitting procedure, as described in Section 3.

### 3. Results

It is well known that to obtain the force-versus-indentation curve of a soft sample, the force-versus-distance curve have to be corrected with the force-versus-distance curve measured with the same cantilever on a hard sample (Weisenhorn et al., 1993). Therefore, in the first step of the analysis, the calibration curve  $F_{\text{calib}}(x)$  was inspected (Fig. 2), where  $x$  is relative position of the cantilever. In the model case, the deflection of the indenter on the substrate should be a linear function of applied force and that is indeed the case, but only for relatively significant

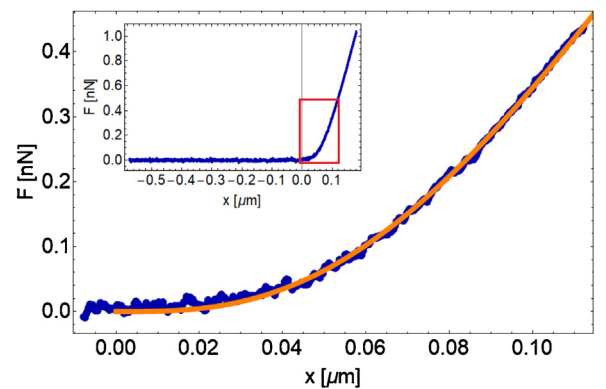


**Fig. 2.** Calibration curve: blue points correspond to the experimental data, while the continuous orange line represents the fitted analytical form, as given by Eq. (7). The inset shows the data on the extended scale. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

forces. However, for very small forces the relation between the force and the position, i.e.,  $F_{\text{cal}}(x)$  appears to be of a higher order. The effect is observed not only in the presence of cells, but also on a clean glass surface in EGM-2, measured with a new, clean probe. Therefore we suppose, that the nonlinearity is caused by the electrostatic interactions. By careful numerical analysis of that part of the calibration curve, we found that dependence should be that of the third power. Additional analysis of the difference between this approach and linear calibration is discussed in Supplementary materials, Section 1.

The analytical expression that fulfils both the asymptotic behaviours of the calibration curve, would be of the following rational function:

$$F_{\text{calib}}(x) = \frac{x^3}{a \cdot x^2 + b \cdot x + c}, \quad (7)$$

where  $a$ ,  $b$  and  $c$  are parameters to be determined. Performing the series expansion, we obtain that for small indentation exhibits the cubic behaviour:

$$F \approx \frac{1}{c}x^3, \quad (8)$$

while for large indentations the linear behaviour appears:

$$F \approx \frac{1}{a}x - \frac{b}{a^2}. \quad (9)$$

After shifting the approach line to the level  $F = 0$ , third order and first order curves as given by Eqs. (8) and (9), were fitted to the corresponding parts of the calibration data and that procedure yielded the initial values of parameters  $a$ ,  $b$  and  $c$ . Finally, at this stage, the full form (7) was fitted and the result is presented in Fig. 2, showing an excellent agreement.

From the physical point of view parameter  $a$  responds to a photo-detector sensitivity, while parameters  $b$  and  $c$  describe the behavior of the tip near the contact point.

One of the key steps in the analysis is determination of the contact point between tip and the cell. To make the computations precise, the second-order curve was fitted to the part of force-distance data for small interaction forces (nonlinear region). It turned out that this approach to finding contact point caused very small variation of final elastic parameters with the small change of the position of contact point.

In the next step of analysis, the analytic form of the calibration curve was subtracted from the experimental force-distance measurements, performed in the nucleus area of the cell (Fig. 3). To begin with, the inverse function  $F_{\text{calib}}^{-1}$  was calculated. Then, each point from data  $(x, F)$ , was transformed into force-indentation by subtracting from registered distance the indentation corresponding to the same value of force in the calibration curve,  $(x, F) \rightarrow (x - F_{\text{calib}}^{-1}(F), F) \equiv (\delta, F(\delta))$ .

Last, the most sensitive step of analysis was numerical

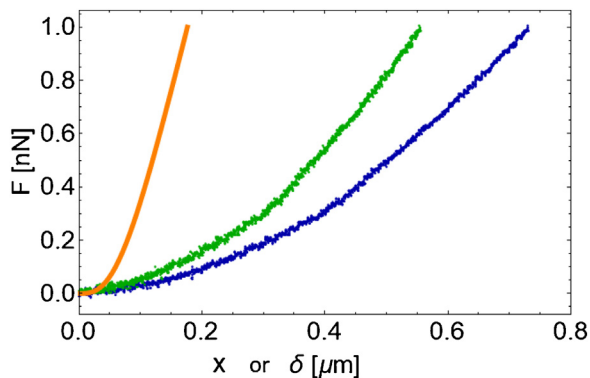


**Fig. 3.** Example of the force-distance  $F(x)$  data: experiment – blue points, calibration curve – orange line, reduced force-indentation  $F(\delta)$  data – green points. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

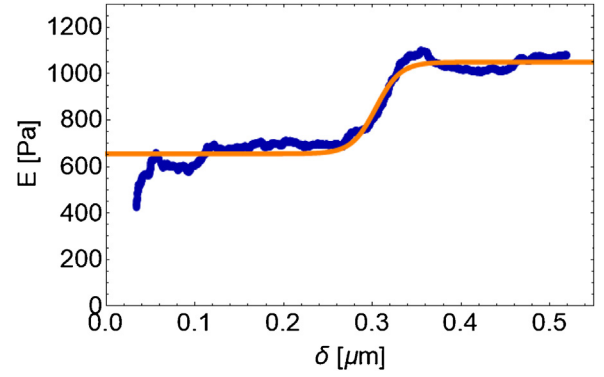


**Fig. 4.** Dependence of the elastic parameter  $E$  on the indentation depth  $\delta$ . Derivative of the experimental data  $E_{\text{exp}}(\delta)$  – blue, model curve derivative, as calculated from Eq. (6) – orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differentiation of the reduced force-indentation curve and fitting (5) and (6) to the obtained data. It is pivotal to emphasise that the local determination of elasticity parameter throughout the sample is accurate and succeeding optimisation with two-layer model has the sole goal of extracting comparable physical parameters. As a result of the fit, the elastic parameter curve  $E(\delta)$  is determined. To avoid numerical lapses it is of the essence to choose the initial values carefully, i.e., the elastic parameters of probed layers. By integrating  $E(\delta)$  curve according to Eq. (6), the force versus indentation curve is retrieved. The examples of differential and integral approaches are presented in Figs. 4 and 5.

Fig. 4 presents the effect of differential approach, that means, from the point-wise computed slope of force-indentation curve the local parameter  $E_{\text{exp}}(\delta)$  from (6) is obtained. Next, taking into account the lowest and the highest values of elasticity parameter, the best fit of  $E(\delta)$  in the form of (5) is established. The model function estimating the changes of elasticity with increasing depth is integrated to regain the model function for force-indentation presented in Fig. 5. The curve which is obtained fits the experimental data correctly for shallow and deep regimes. In our case the first layer corresponds to a cortical layer composed of the cell membrane and the cytoskeleton, and the second one is the cell nucleus.

More examples of analyzed data showing the quality of fit are presented in Supplementary materials, Section 2.

The approach described in Section 2.3 has been used to analyse whole sets of data obtained for mice on control, standard diet (American Institute of Nutrition, AIN/CONT) and high fat diet (HFD). In the process of measurement the  $8 \times 8 \mu\text{m}^2$  maps were acquired, each

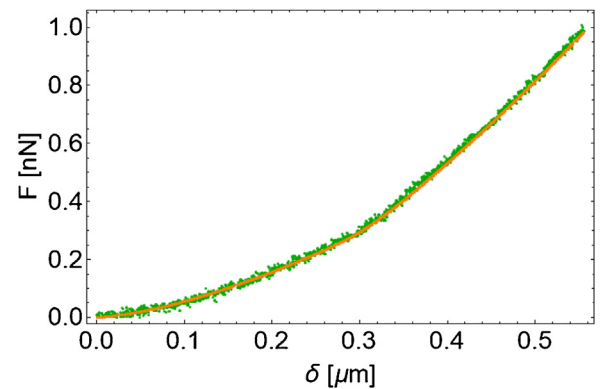


**Fig. 5.** Dependence of the applied force on the indentation depth. Calibrated experimental data – green, model curve resulting from the integration of Eq. (6) with the data presented in Fig. 4 – orange. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

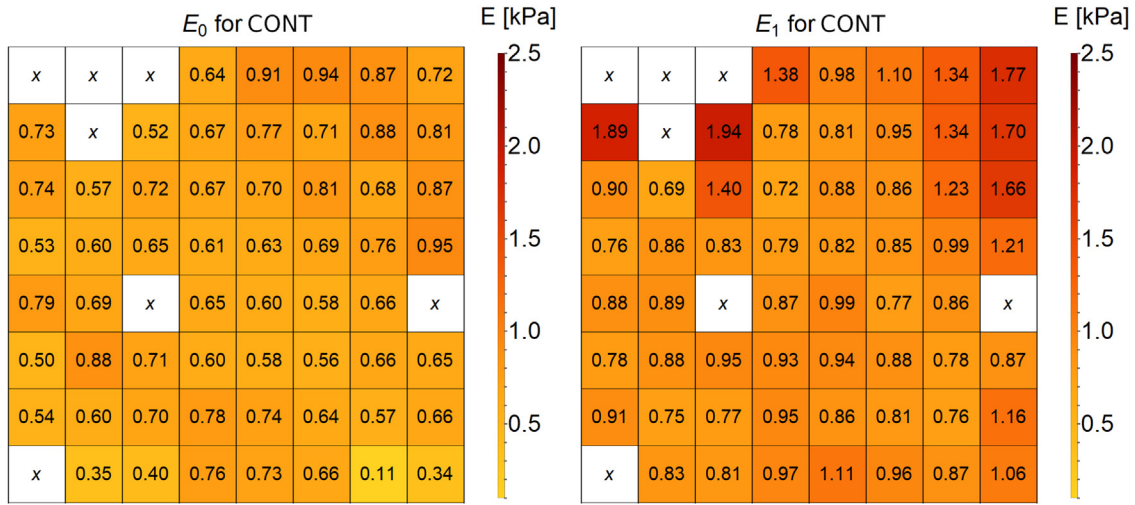


Fig. 6. Map of elastic parameters  $E_0$  and  $E_1$  for CONT cell. Crosses denote curves that were excluded from the analysis.

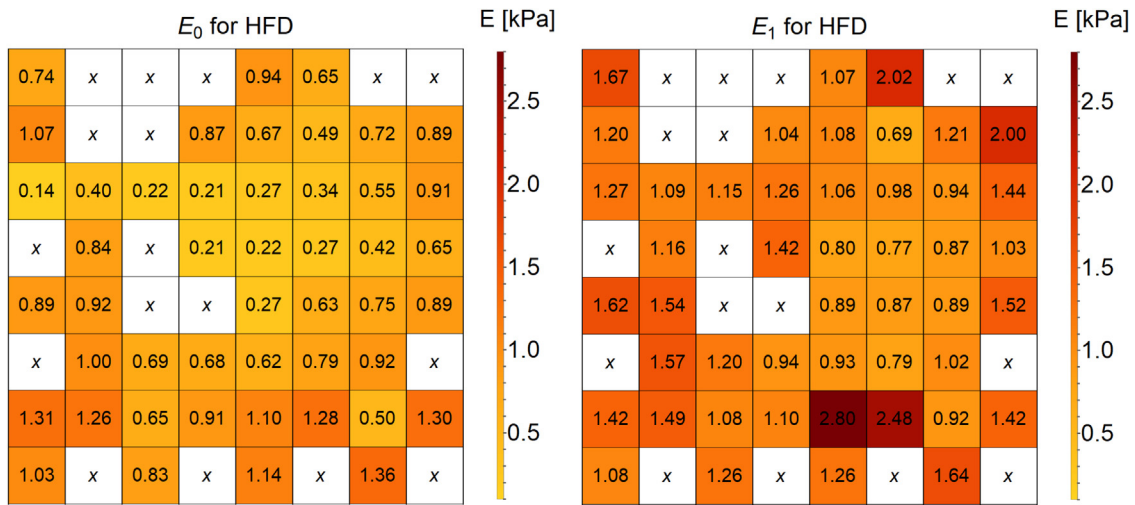


Fig. 7. Map of elastic parameters  $E_0$  and  $E_1$  for HFD cell. Crosses denote curves that were excluded from the analysis.

consisting of 64 force-distance curves. Results are presented in Figs. 6 and 7.

The presented results clearly demonstrate that the local differential approach gives a more detailed information about the elasticity property  $E$  versus the position  $\delta$ . Contrary to that, the commonly used integral approach loses much information yielding the average elasticity properties. Namely, it turns out that the first, outermost layer of the cell is more elastic than the deeper layer ( $E_0 = 570$  Pa and  $E_1 = 880$  Pa). The results for HFD are even higher,  $E_0 = 590$  Pa and  $E_1 = 1040$  Pa for respective medians. What is more, data collected on the edges of the cell nucleus were much more difficult to analyse and some of the curves had to be omitted. The origin of that behaviour is because of the fact that the marginal region seems to be more rigid than central region of the nucleus. When only  $4 \times 4$  map from the very centre was considered the results were  $E_0 = 670$  Pa,  $E_1 = 880$  Pa for CONT and  $E_0 = 450$  Pa,  $E_1 = 980$  Pa for HFD. Interestingly, when similar procedure is applied to classical Sneddon approach, we also observe that curves on the edges of the cell nuclei present much higher Young's moduli (Fig. 8).

Fig. 9 shows the results of analysis of 121 CONT curves and 90 HFD curves from 4 cells using Hertz and double-layer models. In both models the HFD cell are stiffer than CONT cell, however second model demonstrates that the outer layer of the cells is equally elastic in both cases and the difference starts to appear in deeper layers. What is more, careful determination of contact point and application of more complex

calibration procedure slightly increased the value of elastic parameter in comparison to simple use of Hertz model.

#### 4. Discussion

Elasticity is a significant physical property of cells and tissues. While it is investigated with AFM, experimental data have to be analysed and the need for theoretical models arises. So far, for the vast majority of performed and reported studies, the one-parameter models of Hertz and Sneddon have been utilised. The key step in the Hertz/Sneddon approach is the global fitting of an experimental force-indentation curve to a model function, thus providing one effective elasticity parameter. Having performed such an analysis for different systems, the obtained values of the elasticity parameter might be compared, showing changes of elasticity caused by physiological or other factors.

In this report we apply the two-layer model to analyse elasticity parameter of LSECs using two-layer approach, and that is the first use of this model for biological systems.

As a result, we have demonstrated that properties of the biological samples were changing with the depth. That is why the layered model presents the picture which is more relevant, local, and compatible with the actual cell structure. It was indicated by Kochan et al. (2017) using Raman spectroscopy, that changes in LSECs during progression of NAFLD occurred preferentially in the cell nuclei, but not in the

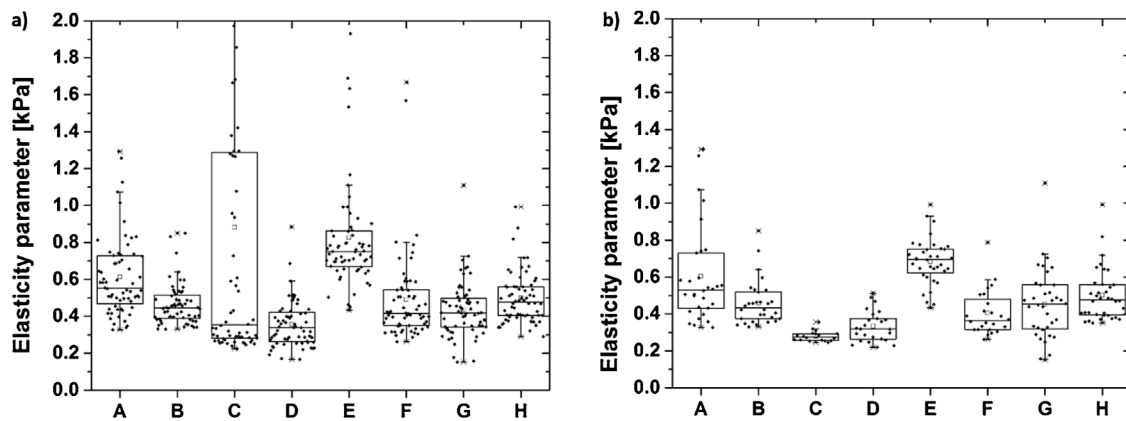


**Fig. 8.** The differences in classical Sneddon approach if only the FD-curves from highest points of reconstructed topography are investigated. Cells A–H presented in Fig. 1 were analysed. (a) presents all the FD-curves from  $8 \times 8$  map acquired in the cell nuclei area, while (b) presents curves from the top of the cell nuclei, i.e., the brightest points in Fig. 1b.

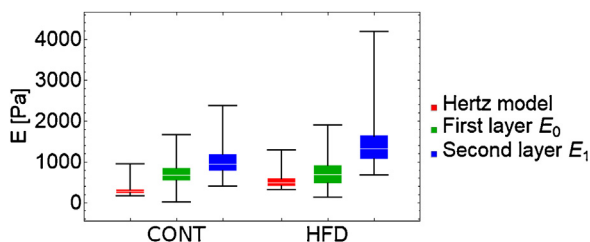


**Fig. 9.** Comparison of the layered model results with simple Hertz model analysis.

cytoplasm. The effect was ascribed to major modifications of the structure of DNA which might influence nanomechanical properties of the nuclear lamina. Our results analyzed using a simple approach with the one-parameter Hertz/Sneddon model failed in providing the specific information about changes in the nucleus elasticity. However, the approach based on the two-layer system allowed us for revealing more complete information, which was in agreement with the results from Raman spectroscopy. The changes in elasticity of the cortical layer in LSECs from control and NALFD groups were not seen, whereas the elasticity of the deeper layer, ascribed to the cell nuclei, was altered in accordance to the Raman spectroscopy results.

One parameter Hertz-Sneddon model have substantial limitations and for more complex systems, it is necessary to allow for at least non-uniform structure of the investigated systems. A layered structure appears as a natural candidate for such an extension. In the simplest case, a two-layer system is considered and that is the model we employ in this line of research. From a mathematical point of view, the model has four parameters, i.e., two limit values of the effective elasticity parameter and two geometrical characteristics that determine the position and the width of the region where elasticity jumps between these two values. In this way, it allows for taking into account the layered structure of biological systems and their elasticity. Moreover, when a derivative is calculated for the experimental and theoretical model data, it allows for local analysis of elastic properties, thus being an extension to the global, i.e., integral approach of Hertz/Sneddon. As a consequence, the elastic parameters of layered biological systems may be studied and we demonstrate that additional effects, not visible within the Hertz/Sneddon scheme, are available for studies.

Proposed method of analysis is also more complex than simple Hertzian estimation of elasticity parameter. Namely, it takes into consideration the calibration curve more precisely, especially in the critical area next to the contact point. Accurate analysis of calibration curve is crucial in determining the elastic properties and other interactions with the outermost layer of the cell.

## 5. Conclusions

A new two-layer model of elasticity was employed, revealing latent effects, not detectable within the traditional one-parameter Hertz/Sneddon approach. By applying the two-layer approach, we obtained the local variation of the elasticity parameter and that enables much more detailed elasticity analysis. The model was used to analyse the data from the AFM measurements of elasticity in liver sinusoidal endothelial cells. The presented results show that the simple one-parameter Hertz/Sneddon model may be insufficient for interpreting elasticity of different areas of LSECs. These cells have thickness below  $3 \mu\text{m}$  therefore the influence of the stiff glass substrate is substantial and obtaining uninterrupted elasticity parameter is hindered.

So far, the two-layer model, as proposed by Kovalev, was applied only to polymers. Our work indicates that the two-layer model of elasticity can be useful for interpretation of the elasticity changes in biological materials. Our data show that despite the lack of differences in the cortical layer, we can see the differences in deeper parts, corresponding to the cell nuclei of LSEC in the non-alcoholic fatty liver disease. The potential of the presented formalism is also related to relative simplicity of extending it to three or even more layers, thus being a natural candidate for the elasticity description in more complex soft-matter systems.

## Acknowledgements

This work was supported by the Polish National Science Centre under the SYMFONIA 3 project, grant agreement no.: UMO-2015/16/W/NZ4/00070.

## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.micron.2019.102705>.

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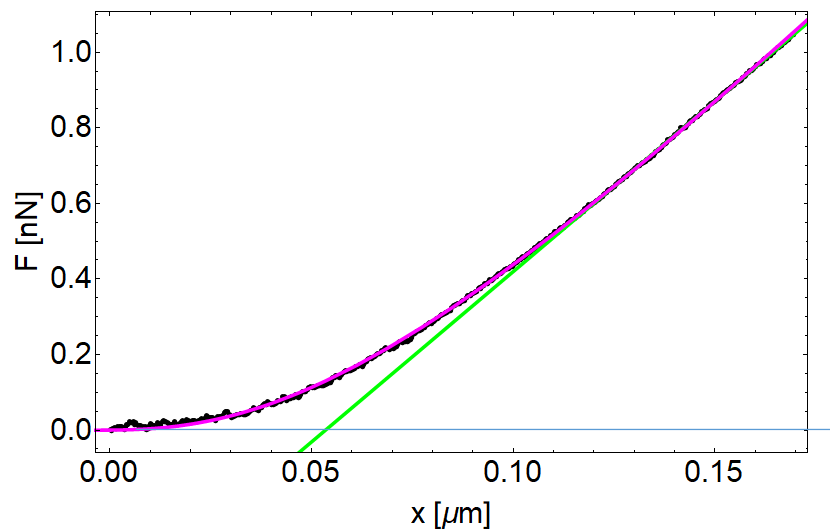
# Application of a layered model for determination of the elasticity of biological systems

## Supplementary materials

M. Rusaczonek, B. Zapotoczny, M. Szymonski, J. Konior

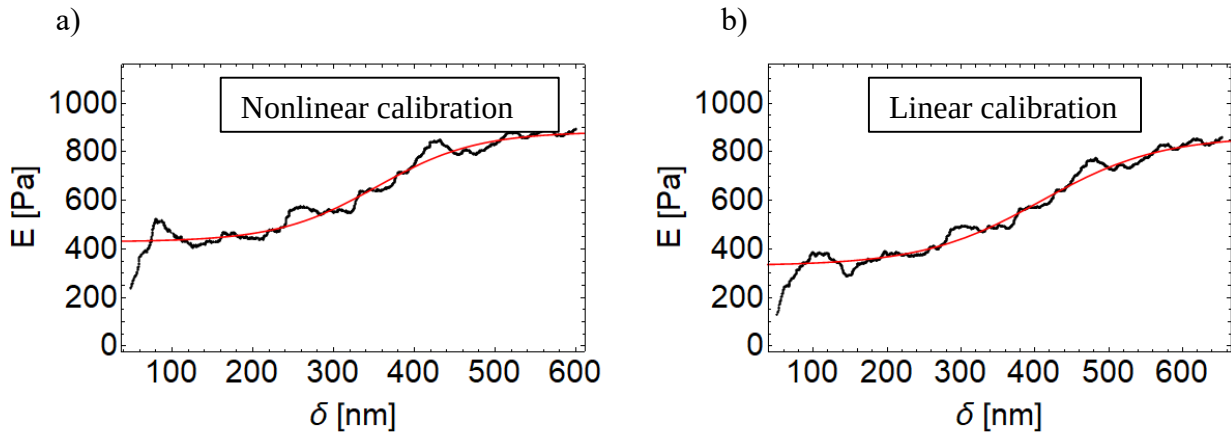
### 1. Comparison of linear and nonlinear calibrations.

We compared our nonlinear calibration with classical linear approach.



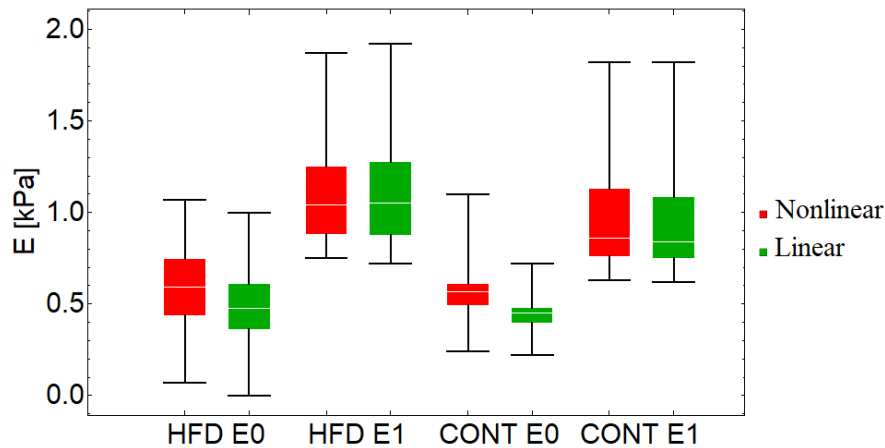
Sup. Fig. 1. Calibration curve fitted with our nonlinear model (pink), and linear approximation (green).

Fitting the calibration curve with a line gives a perfect approximation for larger forces, but suggests different contact point (Sup. Fig. 1.). The question is how the difference in calibration curve influence the results of computing the elasticity parameter. Below, we present the same indentation curves analyzed with our model of calibration and the linear calibration.



Sup. Fig. 2. Fit of two-layer model to data with calibration curve which is a) nonlinear and b) linear.

The value of the elasticity parameter is slightly dependent on the method of calibration, especially – as it was expected – in the first layer, because of the difference in the contact point. However, the crucial results hold. The elasticity of the first layer in CONT and HFD is comparable, while the elasticity of the second layer in HFD remains larger than in CONT (Sup. Fig. 3).



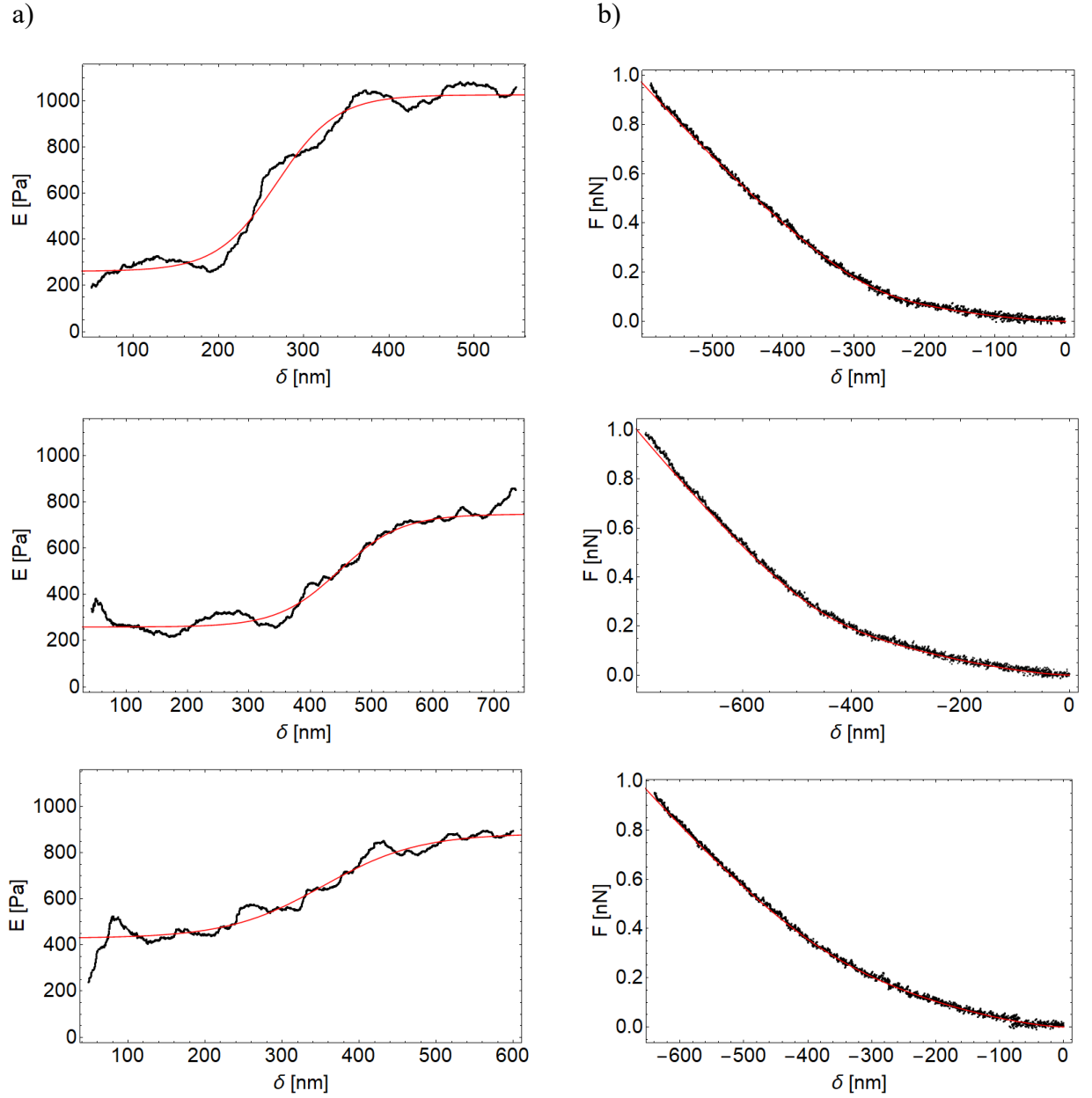
Sup. Fig. 3. Values of elasticity of two-layer model for HFD and CONT with nonlinear (red) and linear (green) calibrations. The data presented here are extension of the results shown in Fig. 9 in the article.

|       | Nonlinear |      | Linear |      |
|-------|-----------|------|--------|------|
| [kPa] | E0        | E1   | E0     | E1   |
| CONT  | 0.57      | 0.88 | 0.45   | 0.86 |
| HFD   | 0.59      | 1.04 | 0.48   | 1.05 |

Sup. Tab. 1. Values of elasticity of two-layer model for HFD and CONT with nonlinear and linear calibrations, taken from Sup. Fig. 3.

Summarizing, the probes showed nonlinear behavior, which was taken into account using more sophisticated method. However, the use of linear calibration would not change the conclusions.

## 2. Examples of elasticity-indentation and force-indentation fits of two-layer model.



Sup. Fig. 4. a) Plots of fitted elasticity parameter as a function of indentation and b) corresponding force-indentation curves with fits.

Endothelial glycocalyx detection and characterization  
by means of atomic force spectroscopy:  
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**Micron**

Volume 151, December 2021, 103153

<https://doi.org/10.1016/j.micron.2021.103153>

Author's contributions

Conducting an analysis using a two-layer model,  
Assessing the potential of using other models,  
Detailed comparison of the models used,  
Contribution to the writing of the article, acting as a correspondent author.



# Endothelial glycocalyx detection and characterization by means of atomic force spectroscopy: Comparison of various data analysis approaches

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## ARTICLE INFO

### Keywords:

Atomic force spectroscopy  
Atomic force microscopy  
Fluorescence microscopy  
Endothelial glycocalyx  
Elasticity  
Heparin  
Heparinase

## ABSTRACT

In recent years, atomic force spectroscopy (AFS) has been used to detect and characterize the endothelial glycocalyx (eGlx) in *in vitro* and *ex vivo* experiments. Several analysis methods were proposed, which differ not only in the numerical implementations, but also in physical models of glycocalyx description. Therefore, it is difficult to directly relate the experiments performed by different groups. In this work, we compared different models used for quantitative analysis of atomic force spectroscopy datasets recorded for eGlx. To capture glycocalyx at various structural conditions, we used basic enzymatic protocols for glycocalyx removal and restoration in human aortal endothelial cells (HAEC). Nanoindentation experiments for this model system were performed for (i) untreated cells, (ii) for cells after heparinase incubation, which enzymatically removes glycocalyx, (iii) for cells with successive heparin treatment, which partially restores the glycocalyx layer. Analysis of nanoindentation data was performed using different models: (a) a single-layer contact mechanics, (b) a double-layer model contact mechanics, (c) a polymer “brush” two-layer model based on the Alexander – de Gennes theory and (d) a simple single-layer “mechanical spring” model. Although different physical parameters are evaluated in methods (a–d), we show that all approaches revealed similar qualitative changes of the glycocalyx layer, which reflected the processes of glycocalyx degradation and its partial restoration. This paper may facilitate a direct comparison of past and future glycocalyx oriented AFS experiments that are analysed with different approaches.

## 1. Introduction

The endothelial glycocalyx (eGlx) is a brush-like layer that tightly covers the luminal side of endothelial cells (ECs). eGlx plays an important role in vascular physiology. As a mechanosensor, the glycocalyx transmits mechanical signals induced by blood flow to the cell, regulating the vascular tension and intercellular adhesion. eGlx also controls the vascular permeability and regulates the oncotic pressure in the vascular system. There is a number of publications (Liu et al., 2018; Nemoto et al., 2020; Zeng, 2017) concerning the role of the glycocalyx injury in the development of vascular dysfunction like atherosclerosis and hypertension.

Moreover, an intact endothelial glycocalyx may act as a shield preventing host infection, for example by the SARS virus via Angiotensin-converting enzyme 2 (ACE2) (Weidenfeld and Kuebler, 2018). The pulmonary endothelial glycocalyx is important in Acute respiratory distress syndrome (ARDS), where its critical role for heparan sulfate was identified (LaRiviere and Schmidt, 2018).

A standard way to visualize and monitor the changes of the glycocalyx structure (shedding, regeneration or distortion) is to use microscopic methods, like the scanning electron microscopy or the confocal microscopy. However, these methods require the use of sample preparation, such as fixation or staining, which can lead to changes or damage of the glycocalyx. Nanoindentation with an atomic force microscope (AFM) tip is an alternative method enabling to measure the glycocalyx properties in the physiological conditions.

In general, Atomic Force Spectroscopy (AFS) is a technique that captures the relation between the force and the position of the probe interacting with the sample. During the interaction, there are two phases: approach (that describes mainly repulsive forces) and retract (that contains repulsive and adhesive interactions). We focus on the first phase, when the probe gradually indents into the cell. In this approach, the AFM probe directly “touches” or “senses” the endothelial glycocalyx. The result of the measurement is the force-distance curve that carries the information about the interaction. Direct approach with AFS enables to gain the information on the mechanical deformation of the glycocalyx

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<https://doi.org/10.1016/j.micron.2021.103153>

Received 9 February 2021; Received in revised form 7 September 2021; Accepted 22 September 2021

Available online 1 October 2021

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layer during its squeezing (deforming) and to calculate its structural parameters like length, thickness, and stiffness.

The findings presented in the literature show that the characterization of glycocalyx mechanics has significantly broadened the knowledge about cell physiology and dysfunction development (Dragovich et al., 2016; Iyer et al., 2009). Pioneering work on the application of AFS technique to glycocalyx detection in both *in vitro* and *ex vivo* conditions were performed in the Münster group (Oberleithner et al., 2011). For example, they have shown that the increase of sodium concentration induces stiffening of the eGlx with simultaneous reduction of the glycocalyx thickness (Oberleithner et al., 2011). In another work, the nanomechanics of the endothelial glycocalyx in experimental sepsis was studied (Wiesinger et al., 2013). The Münster group analysed the AFS data using a straightforward mechanical spring model, with the output parameters being the glycocalyx stiffness and thickness.

O'Callaghan et al. (2011) performed AFS measurements of the stiffness and heterogeneity of the pulmonary endothelial glycocalyx. They treated the glycocalyx layer in terms of contact mechanics and a two-layer composite mode. The elastic modulus (effective Young's modulus) of bovine lung microvascular endothelial cell (BLMVEC) glycocalyx was determined as a pointwise function of the indentation depth before and after enzymatic degradation of specific glycocalyx components.

What is worth emphasising in the beginning, from the physical point of view, the glycocalyx is not linearly elastic in nature. However, in many cases of soft matter studies, AFS experiments are conducted in near-linear elastic regime (Garcia, 2020) and a rough approximation by effective Young's modulus is acceptable.

Bai and Wang (2012) studied the spatio-temporal development of the endothelial glycocalyx layer and its mechanical properties by *in vitro* method for human umbilical vein endothelial cells (HUVECs). In their work, for data analysis a single layer model based on contact mechanics was used. Marsh and Waugh (2013) considered the endothelium as a two-layer structure (glycocalyx and cell cortex) and used contact mechanics in a two-layer system to calculate the elastic modulus and thickness of the glycocalyx of HUVECs. Another straightforward single-layer contact-mechanics study of eGlx was performed by Dragovich et al. (2016), who studied the glycocalyx mediated nitric oxide production through activation of TRP channels. Tagosz-Korecka et al. used the "brush" model, proposed by Igor Sokolov and co-workers, for a quantitative description of glycocalyx parameters in both *in vitro* and *in vivo* experiments. They observed a breakdown of the endothelial glycocalyx barrier under hyperglycemic conditions. Furthermore, nano-mechanical measurements showed reduction of the glycocalyx coverage and gradual degradation of the length of the glycocalyx during the progression of diabetes and atherosclerosis in a mice model. Rusaczonok et al. (2019) applied a double-layer model of Kovalev et al. (2004) to liver sinusoidal endothelial cells (LSECs). A critical comparison of two methods of glycocalyx description from AFS data was presented very recently by Delgadillo et al. (2020), in the context of glycocalyx ability to limit the adhesion of leucocytes.

In this paper, we compare different methods applied to AFS glycocalyx studies, which differ either in the data analysis procedure or originate from different physical descriptions: (a) a single layer contact mechanics model, (b) a double-layer model, (c) an "entropic spring" brush model based on the Alexander – de Gennes theory and (d) a simple "mechanical spring" model. Nanoindentation experiments were performed for (i) untreated cells, (ii) for cells after heparinase incubation, which enzymatically removes glycocalyx, (iii) for cells with successive heparin treatment, which partially restores the glycocalyx layer.

## 2. Experimental background of the AFS nanoindentation measurement

All AFS experiments, which are focused on the glycocalyx characterization are based on the same general nanoindentation principle,

which is schematically explained in Fig. 1. In the nanoindentation measurement, the microscope probe approaches to the cell and very gently squeezes the cell body. In the experiment, the force  $F$  that acts on the cantilever is determined from the cantilever deflection, after careful calibration, through the relation  $F = kd$ ; here  $k$  is the spring constant of the cantilever. The cantilever deflection  $d$  is sensed by the top surface of the cantilever acting as a mirror for a laser beam, which illuminates a multi-element photo-diode. In this experimental geometry, the actual indentation  $\delta$  (deformation of the examined object) is calculated from the following combination of the piezo-scanner position  $Z$  and the cantilever deflection:

$$\delta = Z_0 - Z - d, \quad (1)$$

where  $Z_0$  is the so-called contact point (CP) position;  $\delta$  and  $d$  are by definition non-negative quantities.

Most reported experiments used a large spherical probe (Guz et al., 2014; Job et al., 2016; O'Callaghan et al., 2011; Targosz-Korecka et al., 2017, 2016), but experiments with sharp probes (Chiou et al., 2013; Ding et al., 2018; Gavara and Chadwick, 2016; Guz et al., 2014; Rico et al., 2005) or even tip-less cantilever (Bartosch et al., 2017) have also been reported. If the cell surface is covered by the glycocalyx, at first, the probe squeezes the glycocalyx layer and then indents the cell body. The deflection of the cantilever during approach and squeezing is recorded as a function of the relative vertical sample/scanner movement, in the form of the so-called force-distance curve or force-indentation curve.

Examples of force-indentation curves recorded for cells with and without a glycocalyx layer are presented in Fig. 1. The difference between curves "1" and "2" is evident. In the curve "1" one can recognize two distinct regions that correspond to squeezing of two layers with different mechanical properties. The first range, for a small indentation depth corresponds to the squeezing of the glycocalyx layer, whereas the second one, for larger indentation, corresponds to mechanical response of the cell body. Contrary, in curve "2" the whole range can be modelled by one, constant elasticity parameter.

In general, in the nanoindentation experiment we have to consider the cell not as a homogeneous material, but as a complex object containing structures that differ in mechanical properties. In essence, the cell has a multilayer or shell structure. Force – indentation dependence could reveal the mechanical properties of the soft glycocalyx layer or the cortical layer with the actin cytoskeletal net, or the cell bulk and the cell nucleus. Hence, the mechanical profiling of the cell depends on the indentation range determined by the force used to squeeze the cell. In the simplest approximation, as is shown in Fig. 1, from a single force-indentation curve, one can obtain information about the glycocalyx layer, as the first squeezed layer (region I in curve "1" in Fig. 1), and about the elastic properties of the cell cortex (region II in curve "2" from Fig. 1).

The problem occurring in the analysis of the indentation data is that the mechanical (elastic) properties of the successive layers could interfere. Consequently, the analytical model used for analysis should separate the response from consecutive "mechanical" cellular layers, like the glycocalyx brush and the cell body.

## 3. Mechanical models of glycocalyx in the context of AFS measurements

In the literature, one can find several different approaches for the analysis of nanoindentation data, which focus on detection and characterization of the glycocalyx layer. One of the most important assumptions of all analysis methods is that the glycocalyx is a very soft structure and the signal from the glycocalyx layer is contained in the first part of the force-distance curves, corresponding to the small indentation depth.

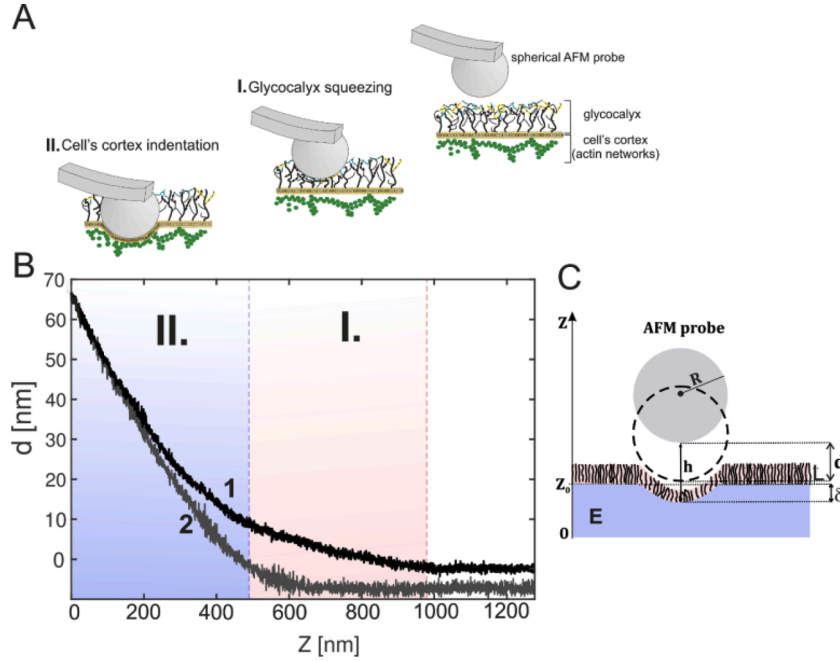


**Fig. 1.** Principle of nanomechanical detection of endothelial glycocalyx with an AFM probe. (A) Schematics of nanoindentation process. At shallow depths the probe senses the glycocalyx layer (region I) and at larger indentation depth also the cell body (region II). (B) Typical nanoindentation curves recorded at locations with (“1”) and without (“2”) glycocalyx. (C) Definition of geometrical parameters.

### 3.1. Contact mechanics - single layer model

Several papers (Marsh and Waugh, 2013; Targosz-Korecka et al., 2017) discuss the glycocalyx layer in the frame of contact mechanics that resembles the Hertz model. In the Hertz model, the force  $F_H$  required to “indent” the body by an indentation depth  $\delta$  using a spherical probe is given by the following expression (Carl and Schillers, 2008):

$$F_H(\delta) = \frac{4}{3} E_Y \sqrt{R_e} \delta^{3/2} \quad (2)$$

where  $R_e$  is the effective probe radius and  $E_Y = E_{glx} / (1 - \nu^2)$ , where  $E_{glx}$  is the effective elastic modulus of the glycocalyx layer and  $\nu$  is the Poisson ratio (Fig. 3A). We perform two separate fits using Hertz-like function given by Eq. (2): one for the regime near the contact point, second for larger indentations of the cell body. The approach of fitting Hertz model to different segments of force-indentation curve in case of cell mechanics was previously discussed by Roduit et al. (2009). In the limit of very small intervals, this technique is called stiffness tomography.

Description of a brush or polymer-like glycocalyx layer using a contact theory is non-intuitive. Glycocalyx should not be described as an isotropic elastic body. However, this approximation is quite frequently discussed and applied in polymer science.

### 3.2. Double-layer model

Glycocalyx covered endothelial cells can be roughly approximated as a two-layer system (Delgadillo et al., 2020). To showcase this approach, the recent local analysis of two-layer system by Rusaczek et al. (2019) was adopted to system of glycocalyx covering the endothelial cell body (Fig. 3B). In this case, both components are treated as elastic materials with elastic parameters  $E_0$  (glycocalyx) and  $E_1$  (membrane with cell cortex). The first layer ends sharply at depth  $\delta_0$ , but the elasticity changes gradually between boundary values, and the transition zone width is described by the parameter  $\alpha$  and that leads to the following equation for the net elastic modulus:

$$E(\delta) = E_0 + \frac{E_1 - E_0}{1 + \exp[-a(E_1 - E_0)(\delta - \delta_0)]} \quad (3)$$

Using a rough approximation that the contact area  $A$  changes with indentation according to Hertz formula, i.e.  $A = \pi R \delta$ , the general relation between surface stiffness and the elastic modulus is given by:

$$\frac{dF}{d\delta} = 2\sqrt{R\delta} E(\delta) \quad (4)$$

Fitting Eqs. (3) and (4) to the data allows to determine the parameters  $E_0$ ,  $E_1$  and  $\delta_0$ .

### 3.3. Brush model

A more rigorous way of analysis of the glycocalyx was proposed by Sokolov et al. (Iyer et al., 2009; Sokolov et al., 2013, 2012). This approach originates from the brush-like structure of the glycocalyx and uses the Alexander-de Gennes (A-dG) theory of polymer brush compression (de Gennes, 1987). In essence, it takes into account an “entropic spring” character of steric forces during glycocalyx squeezing. In A-dG theory, the force required to squeeze a brush of length  $L_{glx}$  to a thickness  $h$  can be approximated as:

$$F_B(h) = 50k_B T N_{glx}^{3/2} L_{glx} \exp(-2\pi h/L_{glx}) \quad (5)$$

where  $k_B$  is the Boltzmann constant,  $T$  is the temperature, and  $N_{glx}$  is the brush grafting density (Fig. 3C). Note that in this paper we use different symbols  $L_{glx}$  and  $T_{glx}$  for description of the same physical quantity, i.e. of the average or model length or thickness of the glycocalyx layer from different models (“brush” vs. “mechanical spring” models). This diversification is only made to emphasize that these quantities are derived from different models.

The “brush” model of Sokolov et al. (Sokolov et al., 2013) allows for a simultaneous derivation of the cell elastic modulus and determination of the glycocalyx parameters from a single indentation curve. In the case of a brush covering the cell surface, Eq. (1) takes the form:

$$\delta - h = Z_0 - Z - d, \quad (6)$$

In the actual experiment, the parameter  $h$  is related to the separation between the AFS probe and the surface of the endothelium covered by the glycocalyx. In the fragment of an indentation curve near the maximal load, one can assume that the brush is almost completely squeezed ( $h \approx 0$ ), and the values of cell elastic modulus can be extracted using the fit of the Hertz model that is limited to the data points in the region of maximum load. In the second step, the derived value of the cell elastic modulus can be used to derive the forces due to the glycocalyx brush, and a linearized fit in the form of Eq. (5) can be performed to determine the brush parameters.

### 3.4. “Mechanical spring” model

Pioneering experiments on AFS detection of the endothelial glycocalyx were performed at Münster University. They implemented the most straightforward approach for modelling the mechanical properties of the glycocalyx layer and they assumed that the mechanical response of the glycocalyx could be summarized in a single equation, based on the Hook’s law, relating the force  $F$  and the indentation  $\delta$ :

$$F = k_{\text{glx}}\delta, \quad (7)$$

where  $k_{\text{glx}}$  stands for the glycocalyx stiffness (usually expressed in pN/nm). Since Eq. (7) is based on Hook’s law, we will refer to this model as a “mechanical spring” model (Fig. 3D). The analysis procedure is in details described in publications (Oberleithner et al., 2011; Wiesinger et al., 2013), and contains two crucial parts. At first, the “contact point” (CP) is determined in the force-distance curve as a point at which the AFM probe comes into contact with the sample (Gavara and Chadwick, 2016). Second, from the contact point a linear fit is performed to the first part of the curve. This procedure is usually based on examining the  $R^2$  factor of the linear fit. As a result, the value of the slope and length of the first part of the force-distance curve is calculated. Value of the slope corresponds to the glycocalyx stiffness  $k_{\text{glx}}$  and the glycocalyx thickness  $T_{\text{glx}}$  is the length, for which a pre-defined or maximal value of  $R^2$  was found.

## 4. Results and discussion

### 4.1. Visualization of the glycocalyx

Fig. 2 shows the results of glycocalyx staining observed in fluorescence microscopy. Fig. 2(A) was acquired for untreated HAEC cells. Fig. 2(B) and (C) show cells with glycocalyx at different conditions: for cells after heparinase incubation, which enzymatically removes most part of the glycocalyx and for cells with successive heparin treatment, which partially restores the glycocalyx layer. These three cellular systems with different glycocalyx conditions served as a model for comparison of various methods of the AFS data analysis.

### 4.2. Contact mechanics analysis

At first, we compare two analysis models based on the contact mechanics. In the naïve approach to analyse the glycocalyx/cell system, the data analysis is performed using a simple Hertz model, which is independently applied to two different regions of force-indentation curves as shown in Fig. 3(A). This approach is crude, but has been shown to be very fast and useful for preliminary stages of data classification in *in vitro* studies for imaging of the spatial distribution of the glycocalyx (Bai and Wang, 2012; Targosz-Korecka et al., 2017). In tissues, the spatial distribution of the glycocalyx is very non-uniform, with areas covered and uncovered by the glycocalyx coexisting in adjacent neighbourhoods. Therefore, an *a priori* choice of the exact analysis method is just not possible here. The Hertz fit, limited to the low indentation depth, reflects the Young’s modulus of the surface layer. The second Hertz fit, limited to large indentation depths, reflects the cell body elastic properties.

A more complex contact mechanics approach assumes a local depth dependent Young’s modulus  $E(\delta)$ . This quantity is obtained from a force indentation curve, via a derivative calculated according to Eq. (4). In this paper, we assume that the effective Young’s modulus can be described using a two-layer approximation from Eq. (3). This approach has been recently applied to the study of liver sinusoidal endothelial cells structure. A fit of the model from Eq. (3) is shown in Fig. 3(B).

Both approaches (CM – Contact mechanics, DL – double layer) have

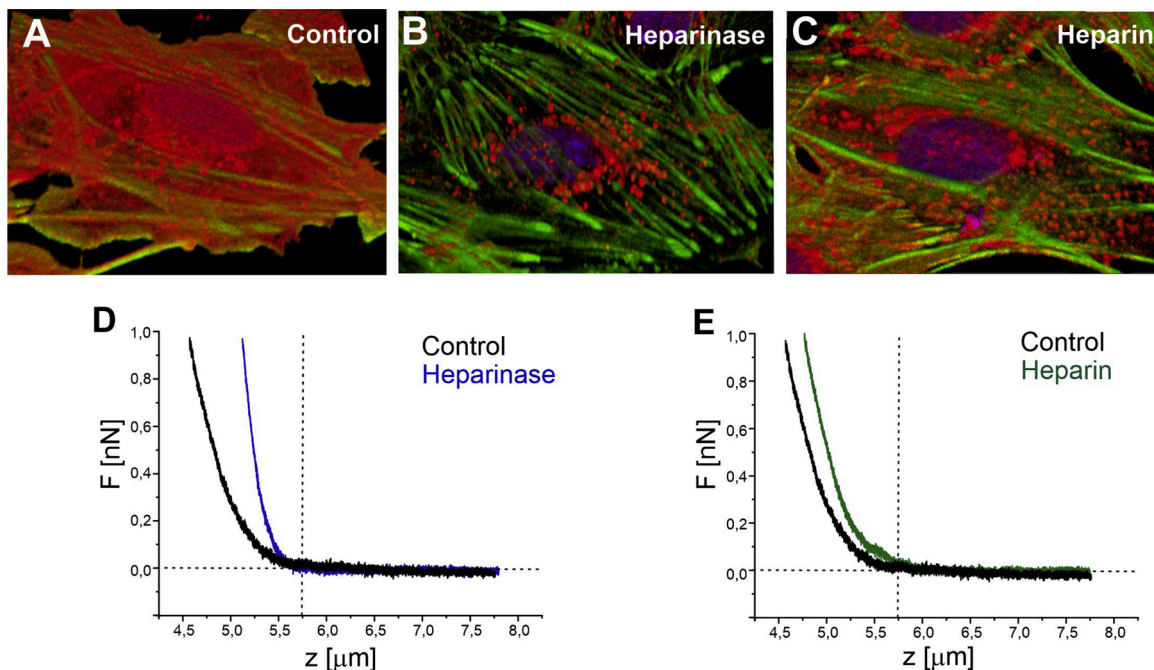


**Fig. 2.** Glycocalyx observed by fluorescence microscopy. (A) Untreated HAEC cells. (B) cells after heparinase incubation – enzymatic glycocalyx removal. (C) Cells with successive heparin treatment, which partially restores the glycocalyx layer. Red – WGA, Green – actin fibre, Blue – nuclei. Comparison of representative force-distance curves measured for control cells and cells treated by heparinase (D) and cells treated by heparin (E).

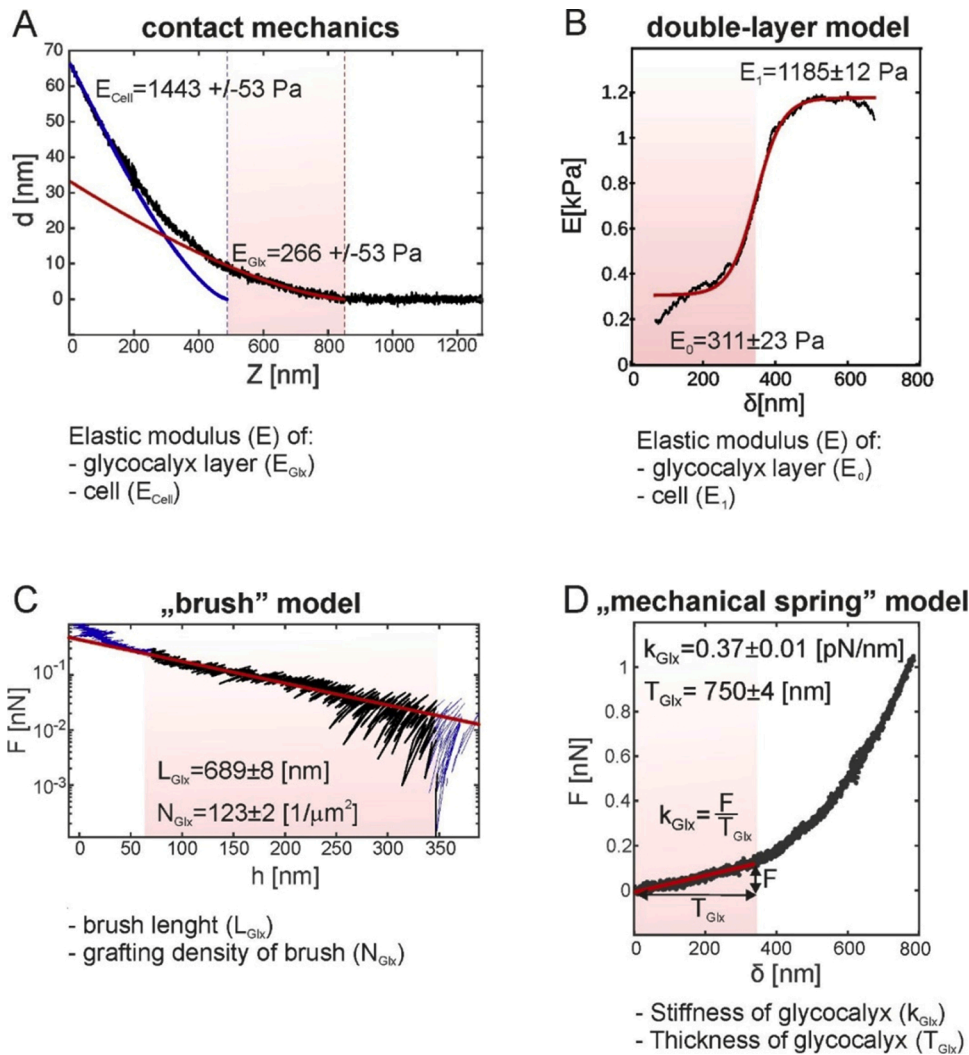


**Fig. 3.** Derivation of the glycocalyx parameters and cell elastic modulus from indentation curves in different models. **(A) Contact mechanics.** The effective elastic modulus of the glycocalyx ( $E_{Glx}$ ) is derived from the Hertz model fit to the data near the contact point. The effective elastic modulus of cell ( $E_{Cell}$ ) is derived from the Hertz model fit to the data near the maximal load. **(B) Double-layer model.** Based on the contact mechanics and the local approach proposed by Kovalev, the elastic modulus of the outside, glycocalyx layer ( $E_0$ ) and the next layer of cells' cortex ( $E_1$ ) are calculated. **(C) Brush model.** Glycocalyx length ( $L_{Glx}$ ) and grafting density is derived according to Alexander-de Gennes theory. This step is preceded by a determination of the cell elastic modulus from the maximal load fragment of the curve as shown in (A). **(D) “Mechanical spring” model.** Stiffness ( $k_{Glx}$ ) and thickness ( $T_{Glx}$ ) of glycocalyx are derived from linear fit to the quasi-linear region of the  $F(\delta)$  curve (near the contact point).

been applied to analyse the data recorded for untreated HAEC cells (Ctr) for cells treated with heparinase (eHep) and for cells firstly treated with heparinase and next with heparin treatment (Hep) in Fig. 4. Left and middle plots shows datasets obtained with simple contact mechanics model and with two-layer approach, respectively. The plots on the right compare the means for the different states of the glycocalyx layer. Young's moduli for both glycocalyx ( $E_{glx}$ ) and for the cell body ( $E_{cell}$ ) are shown.

Application of the contact mechanics to the experimental data has shown that heparinase significantly increases the effective elastic modulus of the first layer - glycocalyx as well as the second layer identified as a cell cortex. What is more, heparin slightly reduces the effects of heparinase by decreasing the elastic moduli. The presented results clearly demonstrate that the effective elastic modulus within the (DL) approach is lower than that obtained within the (CM) scheme. However, when we consider the relative values, in DL model heparinase increases the average effective elastic parameter of the glycocalyx by over 316 %, and heparin decreases the value, so in the end it is only 67 % larger than the control value for that model. On the other hand, in CM model the initial relative increase is smaller, only by 210 %, while after the addition of heparin the elasticity parameter reduces to 45 % more than the control value for that model. In both models we observe the same trend of changes in elasticity.

#### 4.3. “Brush” model vs. “mechanical spring” model

In the next step, we compared the results derived after the application of the models strictly dedicated to analysing the glycocalyx parameters (Fig. 5).

For the control sample, the mean values of all extracted structural parameters are similar to the previously reported values. The mean glycocalyx length  $L_{glx(BM)}$  derived from the brush model is  $(692 \pm 88) \text{ nm}$  and the mean glycocalyx thickness  $T_{glx}$  derived from the “mechanical spring” model is  $(578 \pm 72) \text{ nm}$ . In comparison, Wiesinger et al. (2013), reported glycocalyx length about 300 nm for human pulmonary microvascular endothelial cells (HPMEC). Another experiment performed on-chip (Tsvirkun et al., 2017), gave a value of  $(670 \pm 200) \text{ nm}$ .

Importantly, the derived structural parameters of the glycocalyx unambiguously reflect the degradation of the glycocalyx after heparinase treatment. In general,  $L_{glx}$  from the brush model and  $T_{glx}$  from the mechanical spring model have the same physical interpretation and just correspond to the length or thickness of the glycocalyx layer. However, in all cases we observed that  $T_{glx} < L_{glx}$ . After heparinase treatment both parameters decreased in a statistically significant way, which denotes the effect of glycocalyx “thinning”. However, only  $L_{glx}$  shows a statistically significant increase after heparin treatment and  $T_{glx}$  stays at the same level. The effect of glycocalyx degradation is visible also in the plots, which show the apparent elastic modulus  $E_{glx}$  and stiffness  $k_{glx}$  of the glycocalyx derived from the contact mechanics and “mechanical” spring model, respectively. After enzymatic degradation, both

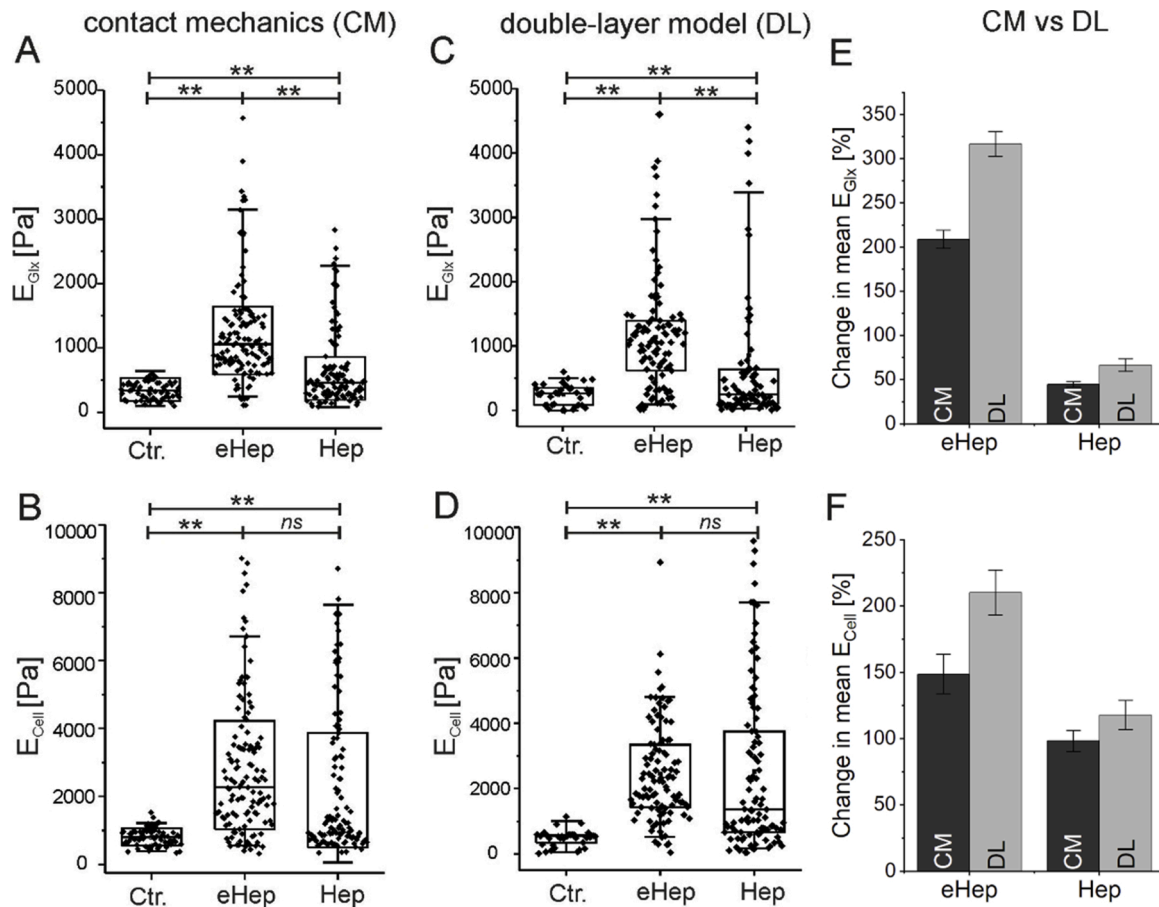


**Fig. 4.** Comparison of the elastic modulus values calculated by fitting the contact mechanics (CM) and double-layer model (DL) to the same data set. The nanoindentation data was obtained for untreated control HAEC cells (Ctr., n = 80 curves, 5 cells) and for cells treated with heparinase (eHep, n = 160 curves, 10 cells) and successive heparin treatment (Hep, n = 128 curves, 8 cells). (A,B) Effective elastic modulus of the glycocalyx layer ( $E_{glx}$ ) and the cell ( $E_{cell}$ ) calculated from contact mechanics. (C, D) values of elastic modulus derived using the double-layer model. (E, F) graphs depict the relative change in mean values derived for the same data set by using the CM and DL models in comparison to the control sample.

parameters increased. This result can be interpreted by the fact that, apparently, the shorter glycocalyx has effectively a higher “stiffness”. After partial glycocalyx regeneration, both parameters almost return to the value characteristic of the control sample.

#### 4.4. Summary of the presented models and their results

To highlight the differences between used models the main information is included in Table 1. The approaches vary in basic assumptions about the structure of the system, in the complexity of mathematical analysis, and in the delivered output properties. Each method allows us to gain alternative insight into the glycocalyx structure.

As for the comparison between the results, the following comments are in order. For the control sample, the mean glycocalyx stiffness  $k_{glx}$  obtained with the “mechanical spring” model has a value ( $0.60 \pm 0.11$ ) pN/nm, and elasticity  $E_{glx}$  obtained for the double layer model is ( $270 \pm 150$ ) kPa and ( $390 \pm 250$ ) Pa for contact mechanics. The obtained value reasonably well agrees with the value of 0.34 pN/nm reported for HPMEC in (Wiesinger et al., 2013). Similarly, the mean value of the effective elastic modulus being below 500 Pa is characteristic of experiments, which used contact mechanics for the description of glycocalyx, for example  $0.26 \pm 0.03$  kPa (O’Callaghan et al., 2011).

As the properties of glycocalyx layer of the control samples agree reasonably well with the values measured by the different authors using various approaches, we can move to the discussion of the results of our experiment. We used four different approaches to obtaining parameters describing mechanical properties of glycocalyx under the influence of

enzymatic removal with heparinase and reconstruction with heparin. The effective elasticity parameters estimated on the basis of contact mechanics and double layers models both show initial stiffening of the glycocalyx layer and later partial recovery to initial state. A similar consistency between models is observed when estimating the length of glycocalyx using the brush and the mechanical string models. Firstly, under the influence of heparinase, the layer of glycoproteins shortens significantly, and the heparin partially reverses this process.

The relative changes in elasticity and length depend on the model, because different approaches assume slightly different character of the glycocalyx layer. Although each model reflects different characteristics of the glycoprotein layer, the relative changes are the same across all approaches.

## 5. Conclusions

In this work, we presented four separate approaches to investigate the influence of heparinase and heparin on the structure and mechanical properties of glycocalyx. The most complex model, including the polymer-like structure of the glycocalyx is the “brush” model. Its results were directly compared to very simplified “mechanical spring” model which assumes that in the brush layer the dependence of the force on indentation depth is a linear function. In both approaches, the trend in changing stiffness and thickness of glycocalyx agrees between the samples. Another group of models were those based on Hertz-like behaviour of the cells. The simpler version dismisses any indications of the thickness of the glycocalyx layer and concentrates on retrieving

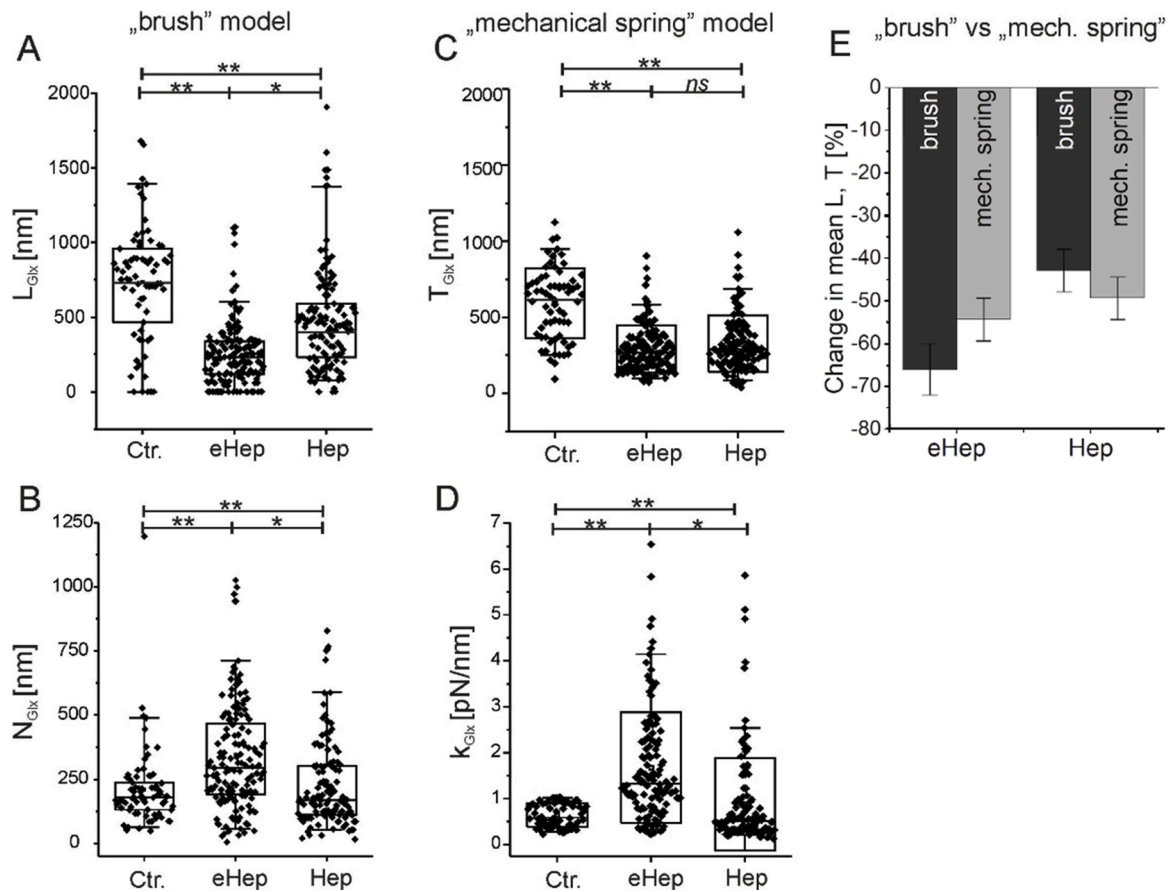


**Fig. 5.** Comparison of glycocalyx parameters derived by fit “brush model” and “mechanical spring” model to the same data set. The glycocalyx parameters were calculated for untreated control (Ctr.,  $n = 80$  curves, 5 cells) HAEC cells and for cells treated with heparinase (eHep,  $n = 160$  curves, 10 cells) and heparin (Hep,  $n = 128$  curves, 8 cells). (A, B) Glycocalyx length and grafting density derived from the “brush” model. (C, D) Glycocalyx thickness and glycocalyx stiffness derived from the “mechanical spring” model. (E) The relative change in mean values of glycocalyx length ( $L_{Glx}$ ) and glycocalyx thickness ( $T_{Glx}$ ) in comparison to the control sample.

the apparent elasticity in the regime of very small and high indentations. More elaborate version, focusses on local elastic properties of the sample. Thanks to that, it is able to predict not only the elasticity but also the approximated length of glycocalyx.

We conclude that all of the presented models give consistent and qualitatively compatible results and they are capable to answer the question about the influence of investigated compounds on the pericellular matrix. With no doubt, heparinase shortens and stiffens the glycocalyx surrounding HAECs. On the other hand, heparin partially reverses the results of heparinase by rebuilding the polysaccharide coating. Nevertheless, to get quantitative conclusions one should be cautious in choosing the proper analysis technique, and should bear in mind all the assumptions and approximations of these models. What is important to stress, the AFS experiments are an indirect method of measuring the dimensions of endothelial glycocalyx. We observe the interaction of the tip with the sample, and deduce the length and stiffness from force-indentation curves.

All presented models are based on different assumptions. Contact mechanics and double layer models are a derivative of the Hertz model, which implies a specific geometry and linear elasticity of the material. The brush and mechanical spring models reflect the different nature of the glycoprotein layer compared to the cell itself. The mechanical spring model is a very simplified approach, while the brush model is based on more complex statistical mechanics and polymer brush theory. We would like to emphasize that the considered models yield different output properties and comparison between them is not straightforward.

## 6. Methods

### 6.1. Cell culture

Human Aortal Endothelial Cell (HAEC) line was grown in Vascular Cell Basal Medium (Cat. No. PCS-100-022, ATCC) supplemented with Endothelial Cell Growth Kit-BBE (PCS-100-040, ATCC). The cells were maintained in standard conditions at 37 °C, 5 % CO<sub>2</sub>, and 95 % humidity.

### 6.2. Experimental scheme

To modify the glycocalyx layer, the model of heparinase-heparin treatment cell was used. At first, the cell was treated with heparinase III (1000U/mL, Sigma) for 1.5 h and next the medium was replaced and heparin solution (1000U/mL, Sigma) was added. The AFM nano-indentation measurements were performed after heparinase and heparin application separately for the same sample.

### 6.3. AFS experiments

The nanoindentation measurements were performed using a Nano-Wizard 3 NanoScience AFM commercial instrument (JPK Instruments, Germany). The spherical (colloidal) probes with diameter 4.5  $\mu$ m (Novascan, USA) attached to the cantilever with a spring constant of 0.01 N/m were used to detect the endothelial glycocalyx and determine the apparent elastic modulus of HAEC. Force-distance curves were

**Table 1**  
Comparison of the applied models.

| Model                          | Fitted parameters                                                           | Advantages                                                     | Disadvantages                                            |
|--------------------------------|-----------------------------------------------------------------------------|----------------------------------------------------------------|----------------------------------------------------------|
| Contact mechanics (CM) model   | $E_{glx}$ – elastic modulus of glycocalyx                                   | Simple analysis                                                | Arbitrary assumption about the positions of the layers   |
|                                | $E_{cell}$ – elastic modulus of cell                                        | Easy to apply                                                  | Vague information about the thickness of glycocalyx      |
|                                | $E_1, E_2$ – boundary values of elastic modulus of the cell with glycocalyx | Elastic parameter changes within layers                        | More parameters must be fitted                           |
| Double-layer (DL) model        | $x_0$ – position of the transition between layers                           | The impact of the deeper layer in taken into consideration     | Small measurement uncertainties influence the result     |
|                                |                                                                             | Local elasticity value is used directly                        | Complex analysis                                         |
|                                | $L_{glx}$ – length of glycocalyx                                            | Consider the nonlinear behaviour of the glycocalyx             | Assumes large difference between layer elasticities      |
| “Brush” model (BM)             | $N_{glx}$ – grafting density of the brush                                   | Precise information about polymer-like structure of glycocalyx | Complex analysis                                         |
|                                | $T_{glx}$ – thickness of glycocalyx                                         | Simplified version of the brush model                          | Less precise info about glycocalyx                       |
| “Mechanical spring” (MS) model | $k_{glx}$ – stiffness of glycocalyx                                         | Clear concept                                                  | Introduces systematic errors due to the simple structure |
|                                |                                                                             | Easy to apply                                                  |                                                          |

collected on the central part of the cell with a constant indentation velocity of 0.7  $\mu\text{m/s}$  and a maximal force of 1 nN. The nanoindentation measurement was performed on living cells at 36,5 °C in HBSS buffer (H8264, Sigma-Aldrich) enriched with 5 mM glucose and 1 % FBS.

#### 6.4. Glycocalyx and cytoskeleton visualization

HAEC cells were grown on a glass coverslip for 48 h. After this time, the heparinase and heparin was added according to the scheme described in 4.2 After the appropriate stimulation, cells were fixed with paraformaldehyde (3.8 %, Sigma) for 10 min. Next, cells were gently rinsed in PBS three times and incubated with blocking peptide solution (Santa Cruz Biotechnology) for 30 min. After this time, they were washed three times in PBS and incubated with biotinylated wheat germ agglutinin (WGA, Vector Laboratories) for 30 min. Next, cells were washed three times and incubated with avidin conjugated with Alexa Fluor 546. Cell nuclei were stained with Hoechst (Thermo Fisher) and actin filaments were stained with Phalloidin conjugated with Alexa Fluor 488. Images were taken using a Zeiss LSM710 confocal microscope and the original software by Zeiss.

#### 6.5. Data analysis

Principles of derivation of the glycocalyx parameters from indentation curves in different models are explained in Fig. 3. For the estimation of “effective elastic modulus” of the glycocalyx, a Hertz fit from Eq. (2) was performed on an initial part of  $F(z)$  curve as shown in Fig. 3(A). This procedure is a simplified version of a piece-wise analysis of the indentation curves described (Targosz-Korecka et al., 2017). The position of the contact point was determined automatically and used in the “spring model”.

For double-layer model, in the first step of the calculation, the local

dependence of the elastic parameter of the indentation is presented in Fig. 3(B). Then the best fit of the transition function of the form Eq. (3) is performed by least-squares method. Finally, the result is integrated using the formula Eq. (4) to return to force-indentation curve.

Fig. 3(C) demonstrated the derivation of glycocalyx length and grafting density from A-dG theory, i.e. according to Eq. (5). The  $F(h)$  dependence was calculated based on the knowledge of the elastic modulus of the cell body, which was determined to a fit from Fig. 3(C) in a region near the maximum load, as described in Ref. (Sokolov et al., 2013).

In the “mechanical spring” model, the stiffness ( $k_{glx}$ ) and thickness ( $T_{glx}$ ) of the glycocalyx were derived from a linear fit to the quasi-linear fragment of the  $F(z)$  curve, similarly as in Wiesinger et al. (2013). Prior to this procedure, the data was smoothed using a moving averaging filter. Linear fit from Eq. (7), was performed for varying number of points in the experimental curve. The length of glycocalyx  $T_{glx}$  corresponded to the fit with maximal  $R^2$  value. The stiffness  $k_{glx}$  was calculated as the slope of this fit as shown in Fig. 3(D).

The models were implemented in MATLAB and Statistics Toolbox, The MathWorks, Inc., and Mathematica, Wolfram Research, Inc. The plots were prepared in Origin, OriginLab Corporation and Corel DRAW, Corel Corporation.

#### Authors’ contributions

MG contributed analysis tools, performed data analysis, co-wrote the manuscript; KEMZ performed sample preparation and nanoindentation measurements, contributed to designing the study; JK supervised the theoretical formalism of double-layer model, participated in the discussions and commented on the manuscript; MTK conceived and designed the study, contributed analysis tools and performed the data analysis, co-wrote the manuscript.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Acknowledgments

Research funded by Minigrant, POB BioS, B.1.11.2020.81, IDUB UJ. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper.

The present study was financially supported by Research Grant Program at Faculty of Physics, Astronomy and Applied Computer Science, Jagiellonian University (grant no. N17/MNS/00004 to Magdalena Giergiel).

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# AFM image analysis of porous structures by means of neural networks

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## **Biomedical Signal Processing and Control**

Volume 71, Part A, January 2022, 103097

<https://doi.org/10.1016/j.bspc.2021.103097>

### Author's contributions

Proposing the method of analysis,

Writing the script for analysis, optimising and testing it,

Taking into account noise and boundary conditions,

Conducting a complete data analysis using method based on neural network,

Dominant contribution to the writing of the article, acting as a correspondent author.



# AFM image analysis of porous structures by means of neural networks

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## ARTICLE INFO

### Keywords:

Fenestrae  
Liver sinusoidal endothelial cells  
Atomic force microscopy  
Neural networks  
Machine learning

## ABSTRACT

In this work, we presented a method of finding and characterising transmembrane porous structures, called fenestrations, with the help of convolutional neural networks. Case studies are performed on high resolution AFM images of murine liver sinusoidal endothelial cells (LSECs). At first, we evaluated different kinds of noise occurring in the LSEC AFM measurements. Next, we proposed a schematic structure of the neural network suitable for our purpose. We examined different loss functions, optimising the accuracy of fenestration detection. Finally, we presented the method of rough calculation of fenestration size distributions.

We demonstrated that the accuracy of this method surpasses 90%. Furthermore, it is fast, not sensitive to the chosen image contrast and fully deterministic. The simple scheme can be easily modified to different objects of interest, which promotes the use of neural networks as a universal tool for the analysis of various kinds of microscopy images.

## 1. Introduction

An experienced researcher, looking through an optical microscope, can easily assess the condition of cells in culture. Several components may indicate inappropriate cell culture conditions or cell differentiation such as changes in cell size, cell shape, interconnection with other cells, or changes in the structure of the cell nucleus [1,2]. Using fluorescence microscopy, electron microscopy or atomic force microscopy more detailed structural analysis is possible [3–5]. What is more, multiple alterations, such as stiffening of cells or even gene expression result in changed cell morphology at the nanoscale [6,7]. Thus, the morphology of cells is one of the key indicators of the condition of cells *in vitro*.

Utilization of cell morphology for scientific purposes requires quantification of data, which should allow for comparison between groups of cells. The cell shape, density and extent of the endothelial glycocalyx, or fenestration number and its planar density have been quantified and compared [1,8,9]. Because of the variance between individual cells in culture, the processing of large amounts of data is

required in order to describe statistically significant changes between groups of interest. Manual comparison is cumbersome, time-consuming and prone to mistakes. Recently, different applications of neural networks have been reported, helping with the (semi)automatic assessment of the different aspects of the morphology of cells [10–13].

In this article, we focus on morphological structures, which are unique for endothelial cells from liver capillaries, namely fenestrae (or fenestrations). Fenestrae are transcellular pores of size in the range of 50–300 nm, which cover 5–10% of the perinuclear area of liver sinusoidal endothelial cells (LSECs). The regulation of the number and size of fenestrae *in vivo* is of great interest to liver research [14]. Fenestrae ensure passive bidirectional transport of solutes and particles (including chylomicron remnants and some viruses) between the vascular system and liver parenchyma [15,16]. With age or in various liver pathologies number of fenestrae decreases, which has implications not only for the liver but also for other systems [17,18].

Fenestrae are investigated mainly *in vitro* [19]. After isolation, LSECs maintained fenestrated morphology for 1–3 days in culture [20]. It was

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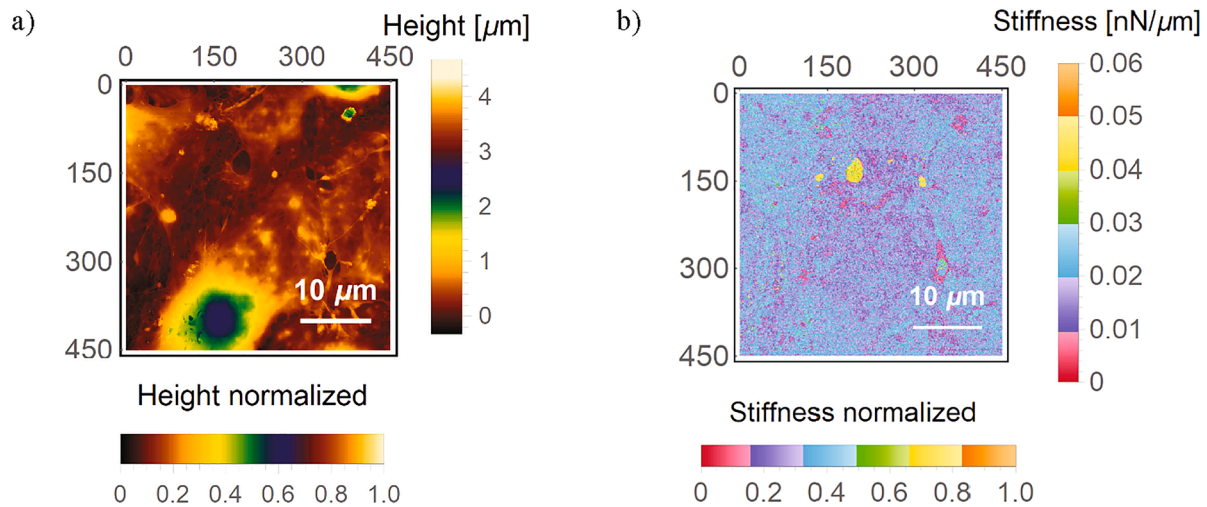


**Fig. 1.** Example of normalization. AFM image of the adjacent LSEC cells with (a) topography and (b) stiffness with raw and normalized data shown by two colour bars. The axes indicate the number of scan lines. The image 450 by 450 pixels covers an area of 40  $\mu\text{m}$  by 40  $\mu\text{m}$ .

reported that fenestrae are actively formed and disappear in minutes and their number and size can be regulated using drug treatment. AFM provides data for both live and fixed cells. For live cell imaging, sequences of individual images collected in the same area with time became frames of animation providing information on time-response of fenestrae to drug treatment [21,22]. LSECs fixed after a certain time of drug treatment, however, provide better statistics for multiple cells measured in the same experimental setup. Moreover, imaging of fixed LSECs is less demanding, because of the increased cell stiffness [23,24]. The AFM images of fenestrae in fixed LSECs have been reported using quantitative imaging mode [22], contact mode [25–27], or peak-force-quantitative nano-mechanical mode [25]. Because of the limited control of the force during imaging and high lateral forces in contact mode, the force spectroscopy based mode provides the best contrast of fenestrae in the obtained images. The main limitation of the method is the time required for individual force curves (pixels of the image) acquisition, which is in the range of tens of milliseconds. Selecting a point-to-point resolution that allows visualization of individual fenestrae results in long-lasting image acquisition ranging from 30 to 60 min per image with resolution 80–100 nm, similar to structured light illumination microscopy [28]. Comparing the size of fenestrae, obtained images have low resolution. In addition, fenestrae are formed in LSEC peripheries, where cell height varies within the range 200–400 nm. Thus, it is hard to define conditions that clearly distinguish fenestrations by means of graphic analysis involving height thresholds and edge detection. With several hundred to a few thousands of fenestrae per image, manual image analysis of fenestrae requires laborious work. Therefore, there is a need for the development of more automatic approaches. Recently, neural networks were reported to model and analyse AFM data [25]. However, the method was applicable only to images presenting part of the individual LSECs, where the image spatial resolution is significantly higher.

Due to their utility, machine learning technics are becoming popular in analysing microscope images [29]. Neural networks were previously used to model and analyse AFM data. For example, a fully-automated neural network analysis of AFM force-distance curves was presented for brain cancer tissue [30]. In other work, artificial neural networks were used for the automated analysis of force map data to determine the local elasticity and to select curves of the best quality [31].

In our work, the neural networks were optimised for finding objects of sizes near the limit of microscope resolution. We present a quick, repeatable and objective method to detect the positions of fenestrations, their occurrence frequency, and information about their size. This work can be also used as a reference for solving different problems, detecting

other kinds of morphological features, and answering various types of questions, as this method is easily modifiable and universal.

## 2. Materials and methods

AFM images analysed in this work were obtained for LSEC samples isolated from mice at the Jagiellonian Centre for Experimental Therapeutics, JCET, in Krakow, Poland, according to a well-established procedure described in [23]. Biological information of the analysed data is not considered in this article.

### 2.1. Cell preparation

After isolation, LSECs were seeded on the uncoated glass coverslips in EGM-2 medium to achieve 60–80% confluence and incubated at 37 °C in the atmosphere of 5% CO<sub>2</sub> for 14–16 h. After that time, cells were chemically fixed using 1% glutaraldehyde for 2 min. AFM measurements on the cells were performed for up to 14 days after fixation.

### 2.2. AFM measurements

Measurements were conducted using Nanowizard 3 AFM system (JPK Instruments). Quantitative Imaging Mode (QI) was used to measure a force curve at each pixel. The maximal loading force of the probing tip was in the range of 250–450 nm, depending on the sample and tip conditions. Thanks to a unique tip movement algorithm, a delicate cell sample was imaged with minimal lateral forces. What is more, various quantities such as stiffness, height etc. could be analysed simultaneously, allowing for constructing of both the topography and stiffness maps of the scanned sample. We used SCM-PIC V2 cantilevers with rectangular tips (spring constant  $k_{\text{nom.}} = 0.1 \text{ N/m}$ ) produced by Bruker. Fixed cells were measured in PBS medium (with Mg<sup>2+</sup>, Ca<sup>2+</sup>) in a commercial liquid cell with the temperature controller (25.0 °C). Two types of images were collected: large images (50  $\mu\text{m} \times 50 \mu\text{m}$ ) with resolution 80–100 nm/pixel and small close-ups (5  $\mu\text{m} \times 5 \mu\text{m}$ ) with resolution 20–30 nm/pixel. Unfortunately, collecting only large images with greater resolution would exceed the available time for performing the experiment. Therefore, we concentrated on analysing images for which pixels were of the size between 83.3 and 90.9 nm/pixel. The pixels were squares ( $\leq 1\%$  difference between x and y dimensions).

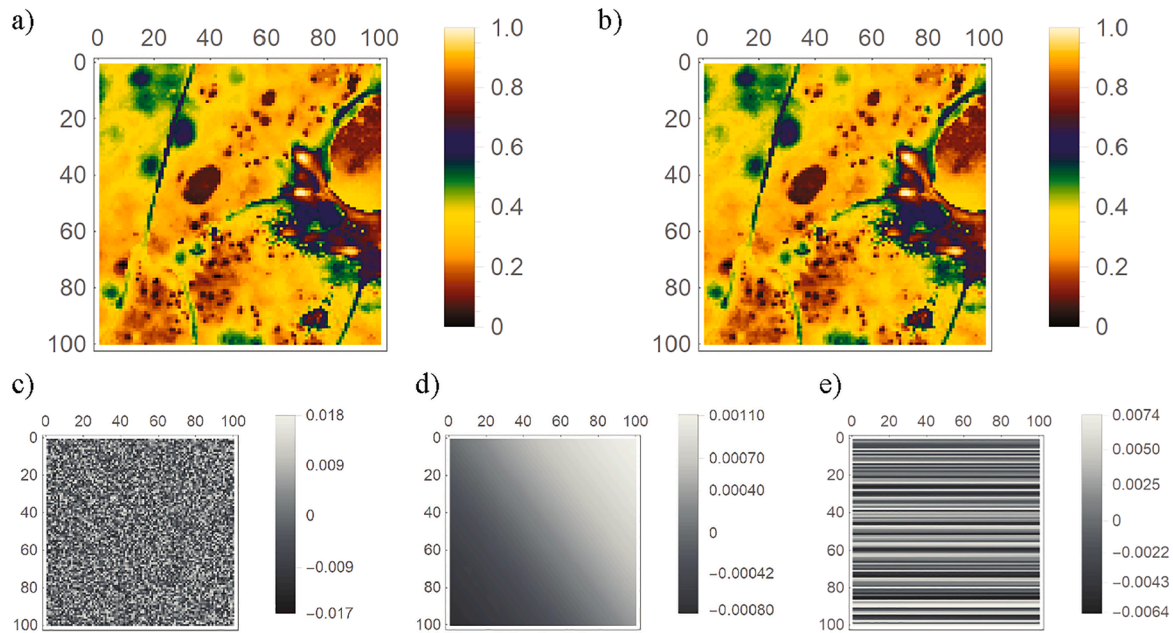


**Fig. 2.** Section of normalized AFM image of LSEC with fenestrations a) raw data b) augmented data with three modifications (c-e), c) random noise, d) random plane, e) random lines. X and Y axes indicate the number of lines in the scan, for (c-e) the height is presented in a grey scale of normalized units (before shifting to zero, to showcase that the change can be positive or negative).

### 3. AFM image processing

#### 3.1. Introduction to creating a neural network

An essential step in constructing an efficient neural network is the proper preparation of the raw data. Firstly, the largest artefacts of the measurements should be removed and then the data are normalized. From the obtained pre-processed data, the training data set is created. It is used to fit the parameters in the model neural network. Training data set contains slightly modified versions of the original data in order to unbiased and enlarge it. After the pre-processing and creating the necessary database, the algorithms of fitting the best parameters to several layers of the neural network are employed. The network is trained and checked both on a training set and the data not used for training. When the results reach the assumed level of efficiency, the network can be used on new data with a high chance of achieving a comparable precision.

#### 3.2. Data pre-processing

As mentioned above, two separate features of the force spectroscopy map were taken into consideration: height at 90% of the maximal indentation depth and stiffness (slope) at the last 10 nm of the indentation curve. Of those two aforementioned, the map of height topography is of greater importance in the process of fenestration recognition. We load raw AFM data, with no pre-processing done prior to our analysis to remove dominant artefacts caused by the shape of the piezoelectric material, thermal drift, or lateral forces [32]. All of those effects were compensated by the neural network – thereby the method provides “end to end” solution almost from raw data to quantitative results of the analysed features. JPK QI files – as computer format TIFF files – were directly loaded into our program written in Python programming language [33].

#### 3.3. Normalization

We normalize the AFM height topography data to take values from 0 to 1. We rescale the physical data to dimensionless values keeping the conversion rate universal for all images (see Fig. 1).

Firstly, we approximate the maximum height in the data set and recalculate the values from the image layers with the linear transformation. In our case, the most important information is stored in the region near the surface. We use the same transformation for all images (the lowest pixel value always starts at 0).

The mean of maximal image height throughout all samples was  $2.70 \mu\text{m}$  (with standard deviation  $\sigma = 0.66 \mu\text{m}$ ). Therefore, the maximal value for the normalization of height is taken as ‘mean +  $3\sigma$ ’ =  $4.68 \mu\text{m}$ . The histogram of maximal values (excluding sample impurities and artificial streaks) is presented in the [supplementary materials](#) section. A similar procedure was conducted for the stiffness (the maximal value was  $0.06 \text{ nN}/\mu\text{m}$ , see Fig. 1b).

#### 3.4. Creating the training data set

The training data set is a collection of slightly modified data with added noise and disruptions, as described below. They are based on the real distortions, such as thermal drift etc., occurring in the experiment. We use this data augmentation [34,35] for two main purposes: to increase the amount of data used to train the neural network, and to remove unwanted biases from the training set.

For the first goal, the transformation of the “up-down” reflection type is used. This is the only spatial transformation that preserves the physical dimensions and takes under consideration that the lines are always measured in a horizontal direction in the fast axis (tip and scan geometry). For the second purpose, the height layer of data was augmented in the following way.

The first transformation is the addition of small white noise. This is responsible for the consideration of random factors in the experiment. The value of the noise is estimated from the experiment by the variance of height on a glass substrate. The square root of variance on a flat substrate equals 0.13% of the normalized height, which corresponds to 6 nm. The estimation of the white noise amplitude is presented in the [supplementary materials section \(Sup. Fig. 2\)](#).

To avoid relevant information loss, we add only tenth of this, i.e., 0.6 nm white noise. In the cross-section of fenestrations their depth varies from at least 50 nm to more than 150 nm, therefore the added white noise is of much smaller magnitude than the features of interest in the

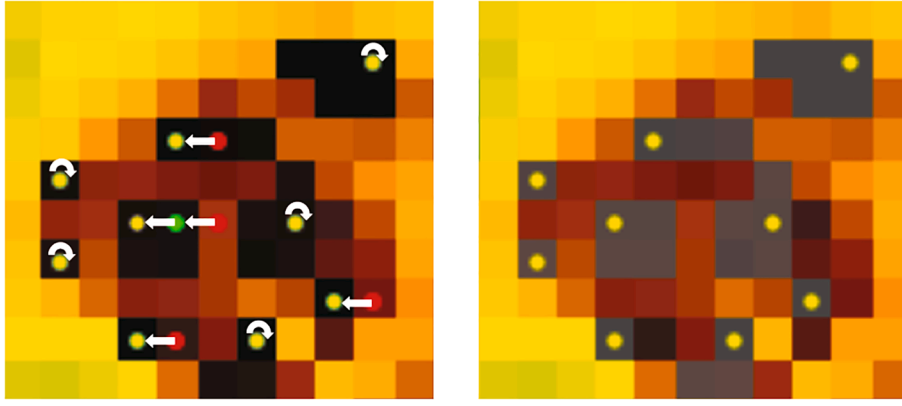


Fig. 3. Moving markers (red-initial, green-middle step, yellow-final position) and flooding area (grey).

image (Fig. 2c).

Next, in the established preprocessing of the data, low-order two-variable polynomial is fitted to part of the data measured near the glass surface. This is done, for example, to compensate for the shape of the piezoelectric material. In short, the AFM image of the flat surface might be a little inclined. To ensure we are not fitting neural network to particular measured inclinations, we add randomly tilted planes to the training images. In our case, we augment the training data set with copies of images, that are shifted by a plane with coefficients  $a, b$ :  $ax + by$  (Fig. 2d). The coefficients  $a$  and  $b$  were estimated as follows. Based on the quick visual analysis we determine that the flat glass surface and the thinnest, flattest parts of the cells deposited on it are mostly present up to the height of about 200 nm. The mean slopes were:  $a = 0 \pm 0.77 \text{ nm}/\mu\text{m}$ ,  $b = 0 \pm 0.70 \text{ nm}/\mu\text{m}$ . Therefore, for the purpose of augmentation,  $a$  and  $b$  parameters were randomly selected from the interval  $(-0.7 \text{ nm}/\mu\text{m}; 0.7 \text{ nm}/\mu\text{m})$ .

Lastly, low-order polynomial is fitted to a part of each fast scan line [32]. This is done, for example, to compensate for the thermal drift in each line separately. We augment the training data set with copies of images, in which each line is shifted independently by some small random linear transformation  $a'x + b'$  (Fig. 2e). Each line shift parameters  $a'$  and  $b'$  are taken to be uniform random variables from the intervals  $a' \in (-0.75 \text{ nm}/\mu\text{m}, 0.75 \text{ nm}/\mu\text{m})$ ,  $b' \in (-25 \text{ nm}, 25 \text{ nm})$ , which are based on the standard deviation estimated from fitting  $a'x + b'$  to scan lines measured near the glass surface.

After adding all augmentations, we shift the data to zero. All those added data points enlarge the training set and help to avoid overfitting the neural network to features of the image, which carry no relevant physical/biological information. All those transformations are shown in Fig. 2.

### 3.5. Positions and area of the fenestrations

The positions of fenestrations in AFM measurements used as training data are tagged by hand. Manual tagging is unfortunately subjective, error prone, and extremely time consuming, because requires marking thousands of fenestrations per image. The two main types of errors are omitting some fenestrations and tagging them in the wrong positions. The perfect marking would be to tag the lowest pixel in each fenestration, as it would be universal and preserved under rotations and reflections.

We adjust the initial positions of fenestration markers in order to increase the neural network learning rate. Nearest neighbours of the marker – one pixel up, down, left, right – are checked (if only they were not the nearest neighbours of other fenestrations). If the height of the neighbour is lower, the marker is moved (see Fig. 3). Such procedure is performed up to 3 times for each marker to make sure that the lowest point was found. In the majority of cases, the marker stays at the initial

position or is moved only one step (it confirms that the manual tagging was done neatly). Most cases in which the marker was at the edge of the fenestrations should now be corrected. Adjusting the markers improves the results of the trained neural network significantly.

Another question that might be asked for characterising fenestrations is a specification of their surface area. However, the accuracy of this measurement is heavily dependent on the resolution. To get the approximate result, for the lowest point of each fenestration, that is found using gradient descent pixel by pixel, one can mark neighbouring pixels of height up to half sigma of the white noise (3 nm) from the bottom of the fenestration. This algorithm is based on the well-known group of flood-filling algorithms [36].

### 3.6. Description of data sets

The data analysis starts with 25 different maps, each with an area between 1000 and 3500  $\mu\text{m}^2$  (excluding nuclei and substrate), the total area of all maps being over 45000  $\mu\text{m}^2$ . The density of fenestrations varies between images from 0.35 to 1.8 fenestration per  $\mu\text{m}^2$ . The total number of tags is 40594. All tags are stored in XML data files (a textual data format, that is both human-readable and machine-readable) containing the position of each fenestration, created with ImageJ [37]. All data were gathered so that each pixel corresponds to the height measured on a square lattice. This way each pixel corresponded to the physical square of size from 83.3 nm to 90.9 nm.

For training, 20 images are used, the remaining 5 are used for testing the accuracy of the network (a testing set). In general, accuracy is computed as:

$$\text{Accuracy} = \frac{\text{True Positive} + \text{True Negative}}{\text{All Instances}}. \quad (1)$$

In our case, we have three classified components (fenestrations, cell membrane, glass surface). We concentrate on the main subject, that is the classification of the fenestrations, providing results on the accuracy of this component.

### 3.7. Description of the network

The choice of neural network architecture is based on the following considerations. Firstly, we want to effectively classify the image assigning each pixel a class: fenestration, cell membrane, glass substrate. Secondly, the smallest features and the ones of greatest significance – fenestrations, have often the size of a single pixel – the architecture has to be able to recognize them and to do so in the context of larger structures they appear in – sieve plates. This means we would like to avoid any loss of spatial data in our network. The natural choice of multiscale architecture similar to “Feature Pyramid Network” [38] could be considered, but we find that the simpler single feature map,

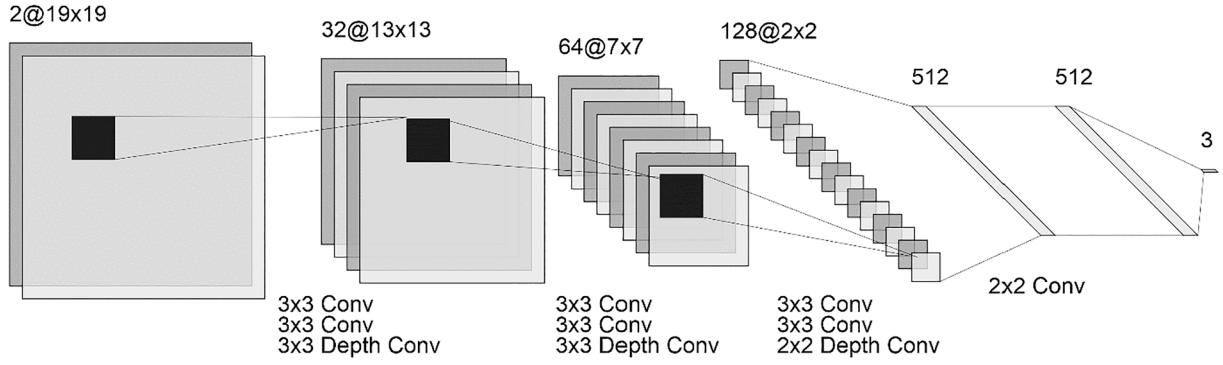


Fig. 4. Schematic structure of neural network. After each block we added a drop-out layer as an additional way to augment data [42].

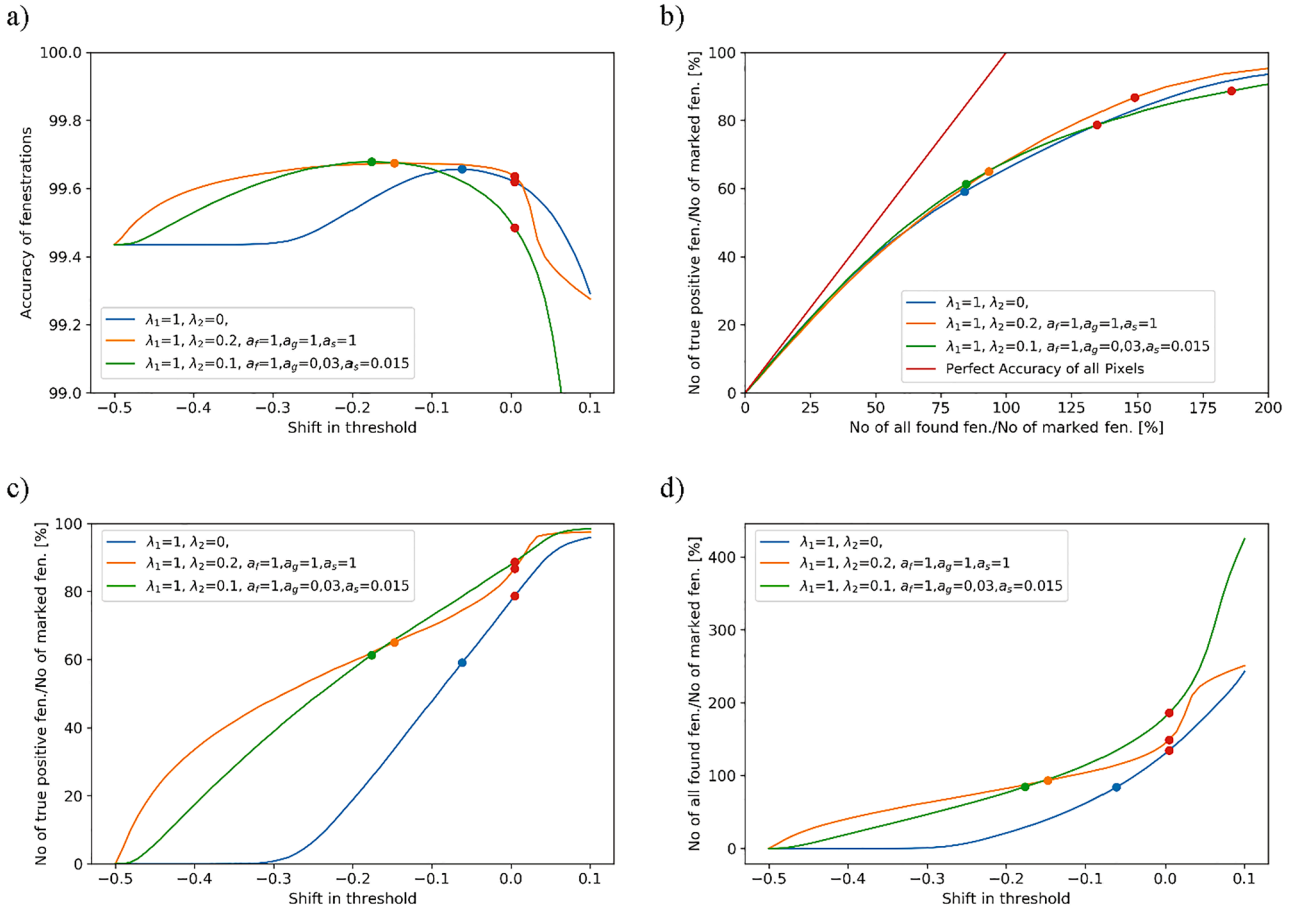


Fig. 5. Classification according to neural network accuracy averaged over all images in training set. a) Accuracy of predicted fenestrations; b) True positive vs. all predicted fenestrations; c) True positive vs. all predicted fenestrations; d) All predictions with different levels of threshold. Red dots indicate the minimal loss function. Green, orange and blue dots show the results for maximal fenestration accuracy. The prediction is considered true positive if it coincides with the flooded area of the original image.

consisting only of convolutional layers with the number of channels increasing with depth, gives already great results that are satisfactory for our purpose. Below, we present the fully convolutional neural network [39] we used, consisting of 12 layers (Fig. 4). The kernels of convolutional layers are of the size  $3 \times 3$  for the first 8 layers,  $2 \times 2$  for the following 2 layers and  $1 \times 1$  for the last two layers. Third, sixth, and ninth layers are depth convolution layers, which output double channels before the next block of convolutions. Network was trained using Adam [40] optimizer with standard parameters: learning rate  $l_r = 0.0002$ ,  $\beta_1 = 0.9$ ,  $\beta_2 = 0.999$  and  $\epsilon = 10^{-8}$ . The training was done on images of size  $22 \times 22$  pixels, for which central  $4 \times 4$  pixels were classified. The batch size

was initially a single such image and was increased in further epochs. Between each epoch, data were augmented. Each epoch data set consisted of images containing every fenestration at least once and additional random images cut out from the measured data. That resulted in the training data in which fenestrations occurred 5 times more likely than normally, which lifted some of class imbalances [41].

### 3.8. Loss function

The loss function is a function that maps values of predicted variables onto a real number representing the accuracy of the prediction. The

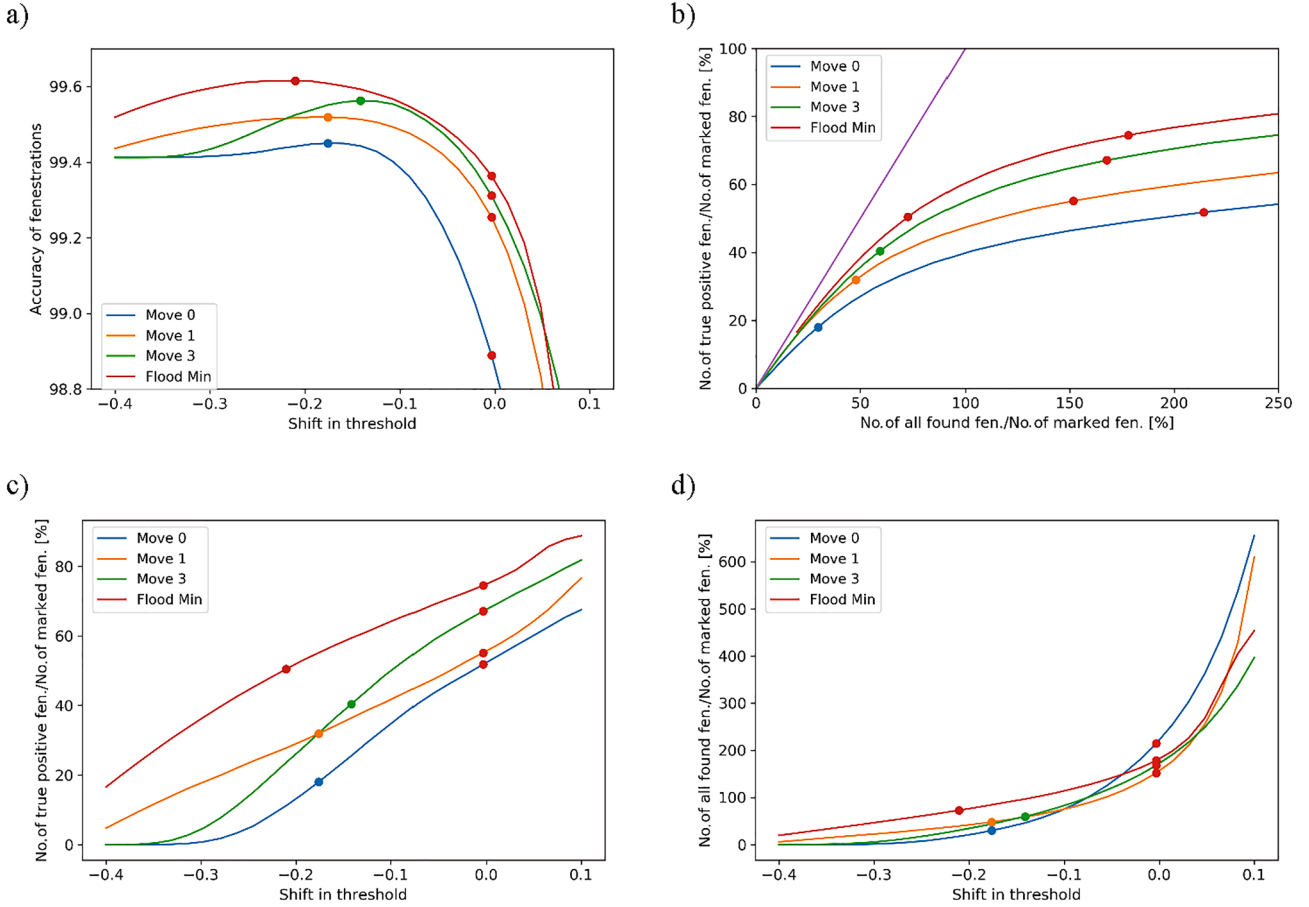


**Fig. 6.** Accuracy of classification of the fenestrations depending on the initial input of the fenestration positions. a) Accuracy of predicted fenestrations; b) True positive vs. All predicted fenestrations; c) True positive with different levels of threshold; d) All predictions with different levels of threshold. Move #, means that initial manually tagged marker was moved by # steps. Flood Min is the minimum of flooded area of fenestration. Bright red dots indicate minimal loss function. Green, orange, blue, and dark red dots show the results for maximal fenestration accuracy.

neural network teaching tries to minimize a loss function.

We propose a loss function with L1 (least absolute deviations) and L2 (least square errors) terms [43], of the form:

$$\lambda_1 \left( \sum_{i=1}^N \delta_{if} - \sum_{i=1}^N p_{if} \right)^2 + \lambda_2 \sum_{i=1}^N (a_f |\delta_{if} - p_{if}| + a_g |\delta_{ig} - p_{ig}| + a_s |\delta_{is} - p_{is}|) \quad (2)$$

where  $\lambda_1, \lambda_2, a_f, a_g, a_s$  are arbitrary coefficients,  $\delta_{if}, \delta_{ig}, \delta_{is}$  are the predictions of neural network for pixel  $i$  if there is a fenestration, glass or cell surface respectively filtered by hyperbolic tangent function. Analogically,  $p_{ix}$ 's are given by:

$$p_{ix} = \begin{cases} 1, & \text{there is } x \text{ at } i \\ 0, & \text{other cases} \end{cases},$$

where  $x$  can be a fenestration, glass or cell surface.

Computations were performed for different sets of parameters. Fig. 5 shows three examples for different values of parameters  $\{\lambda_1, \lambda_2, a_f, a_g, a_s\}$ . For each loss function the neural network was trained for 6 epochs. Fig. 5a. shows a plot for minimal loss function at zero threshold shift. As loss function does not optimize accuracy, the maximal value of fenestration accuracy is reached at different classification threshold (some other value of  $\delta_{if}$ ).

Fig. 5b. presents the relation between all and only true positive predictions. Clearly, by increasing the acceptance threshold more true positive and false positive spots are classified as fenestrations. At the minimum of loss function, the number of fenestrations is overestimated

in relation to those manually marked. On the other hand, for the maximum of fenestration accuracy the number is underestimated by 7–16%. After that point the number of falsely indicated fenestrations overweighs the true positive findings.

## 4. Results

### 4.1. Influence of initial positions of fenestrations

The correctness of the input data for the proper training of the network cannot be overestimated. Therefore, the exact position of fenestration markers must be established in the most repeatable and pedantic manner. Performing this task thoroughly by hand is tedious, if at all possible. What we are left with is tagging as accurately as possible, and then improving results in a standardised way.

Results of the gradual improvement are shown in Fig. 6. We considered the movement of markers by 0 (stayed in place), 1 or 3 steps, as well as placing the marker in the minimum of tagged fenestration. The upswing of accuracy (for all others parameters of network fixed) is remarkable.

### 4.2. Neural network corrective properties

The best network in our study achieved 99.67% average fenestration accuracy in the sense of Eq. (1). This accuracy is stable for 25 different images, measured on different samples. What is essential for the possible utilization of the network is its corrective character. It means that the

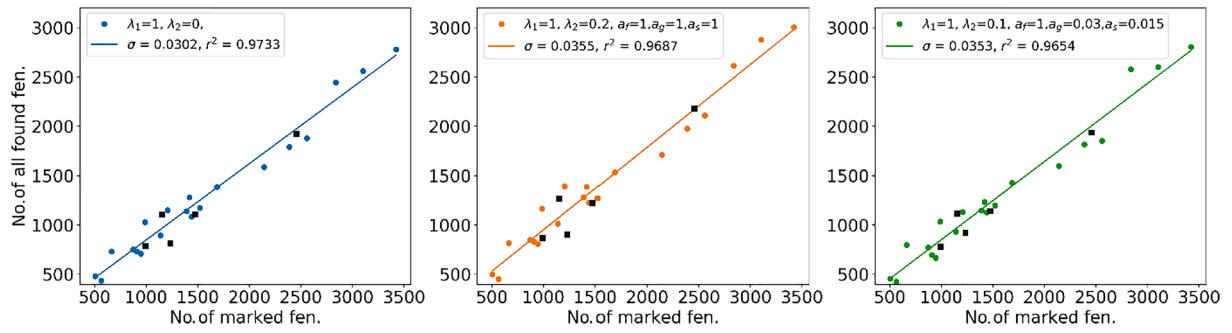


**Fig. 7.** Comparison of corrective properties of neural networks with different loss functions. Dots represent relation between the number of manually marked fenestrations vs. evaluated by network for maximal accuracy threshold on the training set. Black squares represent results for testing set.

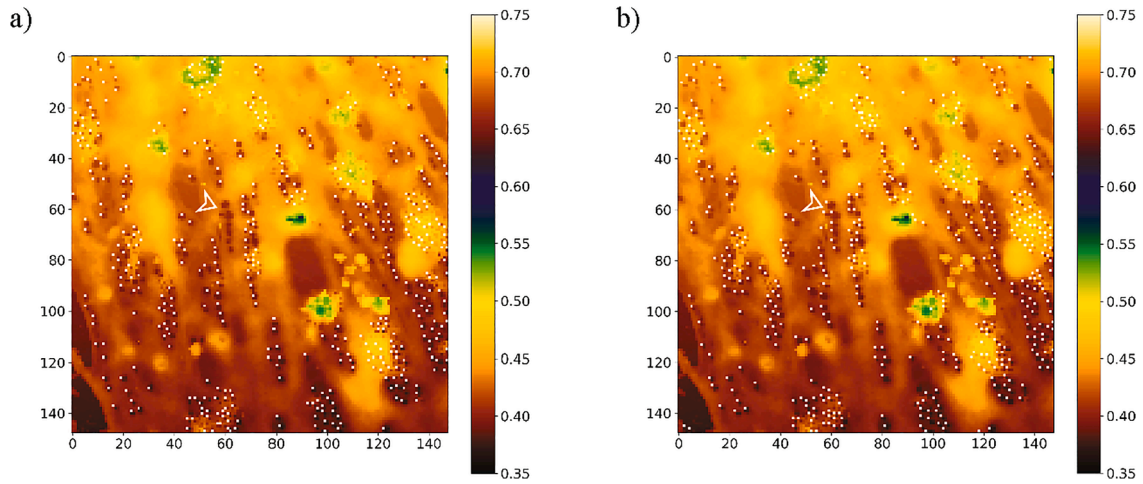


**Fig. 8.** Classifications of fenestrations: a) manual and b) by neural network on the map of normalized height. Axes indicate the number of scan lines, the bar show the colour scale of the normalized image, white dots are the tagged and predicted fenestrations. In the middle of image a) there is a set of fenestrae omitted while marking by hand, however, the network managed to tag them correctly.

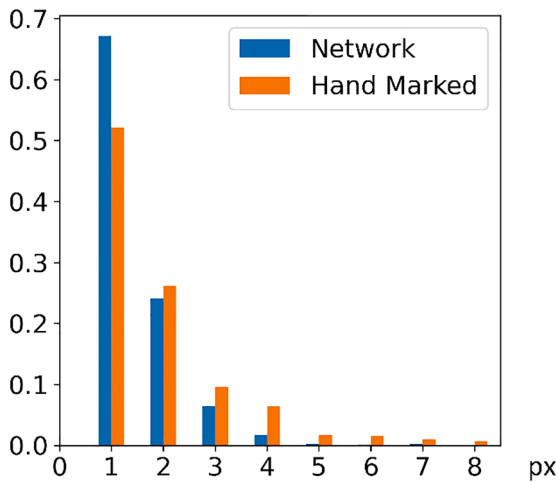


**Fig. 9.** Normalized histogram of occurrence of the fenestration with a certain selected area in pixels (computed for the testing set).

calculated number of fenestrations correlates strongly with the expected value based on manual count. It may be a slightly lower or higher prediction, but for all samples the trend is universal. The results of correlation between the number of fenestrations expected (based on manual marking) and evaluated by the network are shown in Fig. 7.

#### 4.3. Classification of fenestrations

Visual comparison of classifications done manually and with the help of the neural network is quite promising. Typical sieves are classified with appropriate precision, even if they were neglected during tagging by hand. Small artefacts are handled properly. In Fig. 8, there is an example of classification performed on the testing data (they were *never* used for training).

#### 4.4. Fenestrae pixel size distribution

Another difficult task to perform manually is a computation of the fenestration area. Features like mean fenestration diameter and porosity can provide valuable information about the state and properties of sinusoids [8]. Of course, techniques with higher resolution must be provided to ensure more precise, quantitative answers about single fenestrations. However, in this case, even qualitative information averaged from several large images may be useful, for example, to find the largest fenestrations, which can be found using this method. What is more we can estimate the number of holes (gaps), i.e. pores larger than 300 nm that have been described in the literature as a marker of a pathological condition or poor cell condition. In Fig. 9, the histogram of fenestration sizes is presented. The results were obtained in the following way: firstly, the lowest point of each fenestration was found, and then the area up to the height of white noise (6 nm) was flooded. Such an area is considered as an approximation of the fenestration size. We make this approximation, because the fenestration is a pore connecting the glass surface to the cell outer membrane. Therefore, the area of the detected

glass, measured with a sharp tip, could reflect the size of the fenestration [26]. Data in the figure correspond to only one image with about 2400 fenestrations counted manually and 1900 with the help of the network. It turns out that the neural network found one-pixel object more than the manual method, however, in general, there is a quite satisfactory correlation.

## 5. Concluding remarks

We present the neural network approach for finding and counting fenestrations in AFM images. The presented method takes almost raw microscope data and returns a fully classified image within seconds. The results are reasonably accurate and their efficiency is systematically the same among different measurements of cells with differently treated membranes. To achieve this, we extracted different noise terms, that reproduce the pixel variance of AFM measurement and used this knowledge to unbias the noisy data. Such detailed analysis of disruptions is rarely reported.

The size of the training set was quite small consisting of only 20 images. One can expect that building larger dataset of images will lead to better classifiers. Another adversity was the input labelling accuracy, which was imperfect. We analysed the possible correction of initial fenestration positions. Moving the markers towards the lowest point of the pore increases significantly the recall accuracy of the trained network. Additionally, based on flooding approach we present histograms of areas of fenestrations found by manual marking and identified by the network.

Next topic was the influence of different parts of loss function on the trained network. The best results were obtained for a mixed loss function with regression type components L1 (least absolute deviations) and L2 (least square errors). Principally, the problem of finding the minimum of multivariable functions is very difficult, so it is possible that the postulated loss function can still be considerably improved.

Finally, the presented scheme is universal and can be applied to other porous structures, or in general, to local features of the order of a few pixels in size. The use of both height and stiffness as the input opens the door to other possible modifications, such as transfer learning between different microscopic techniques.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Acknowledgements

This work is part of the research project “SYMFONIA 3” supported by the Polish National Science Centre under the grant agreement no.: UMO-2015/16/W/NZ4/00070.

The publication has been supported by a grant from the Priority Research Area DigiWorld under the Strategic Programme Excellence Initiative at the Jagiellonian University.

We would like to thank Prof. Stefan Chłopicki and Dr. Jakub Barbasz for reading the manuscript and helpful remarks.

## Credit author statement

MG contributed analysis tools, performed data analysis, wrote the manuscript; BZ performed nanoindentation measurements, co-wrote the manuscript; ICC performed sample preparation and contributed to designing the study; JK supervised the theoretical formalism, participated in the discussions and commented on the manuscript; MS supervised the study, participated in the discussions and commented on the manuscript; all authors contributed to the manuscript.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bspc.2021.103097>.

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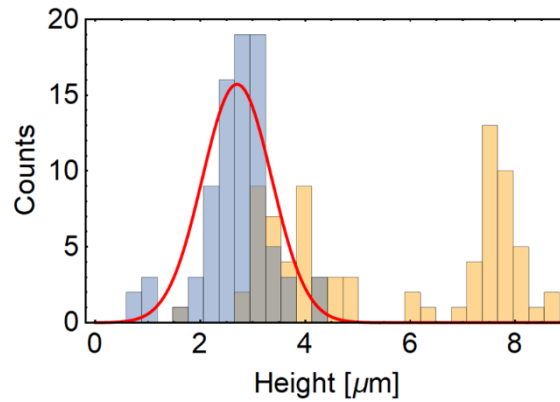
# AFM image analysis of porous structures by means of neural networks

## Supplementary materials

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### 1. Histogram of the maximal value.

Maximum Height Height at 99th percentile

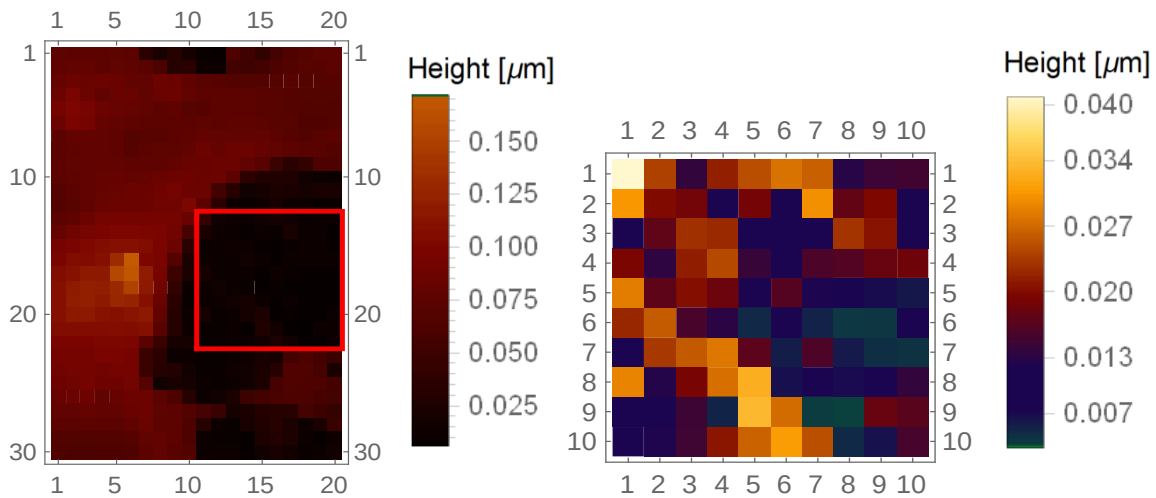


Supplementary Figure 1. Determining the maximum height of the maps for appropriate normalization

In some of the AFM images extra high artefacts occurred. To avoid unnecessary flattening of the regions of importance, the highest percentile of heights was omitted from the calculation of the normalization parameter. In Sup. Fig. 1 we present the histogram of maximal heights computed for 83 AFM images.

### 2. Estimation of the white noise.

One way to estimate the white noise is to compute the variance of height on a flat surface. Three different samples were analysed where the substrate was visible. Square root of variance for a glass is 0.13 % of the normalized height, what corresponds to about 6 nm (Sup. Fig. 2).



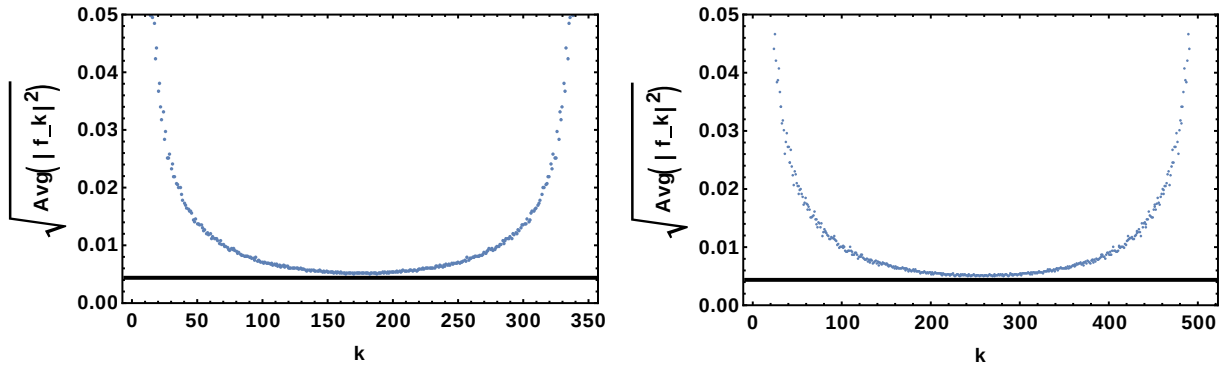
Supplementary Figure 2. Estimation of the noise. The x any y axes indicate the number of scan lines. The colour bars show the height in micrometres.

Another way to estimate the white noise in the experiment is to investigate the Discrete Fourier Transform. Let us assume that in each measured line we have some signal  $f_n$  and some white noise  $\xi_n$  for each pixel in the line  $n=0, \dots, N-1$ ; where  $N$  is the number of pixels in the line. The Fourier transform is linear, so  $F(\{f_n + \xi_n\})_k = F([42])_k + F(\{\xi_n\})_k$ .

By computing the estimated square of the absolute value of the discrete Fourier transform in each line, we can assess the maximum variance of the Gaussian noise (Sup. Fig. 3). In the second step of the calculation we use the fact, that Gaussian noise is uncorrelated:

$$\begin{aligned} E(|\mathcal{F}(\xi_n)_k|^2) &= E\left(\frac{1}{N} \sum_{n=0}^{N-1} \sum_{m=0}^{N-1} e^{\frac{i2\pi kn}{N}} e^{-\frac{i2\pi km}{N}} \xi_n \xi_m\right) \\ &= E\left(\frac{1}{N} \sum_{n=0}^{N-1} e^{\frac{i2\pi kn}{N}} e^{-\frac{i2\pi kn}{N}} \xi_n \xi_n\right) \\ &= E\left(\frac{1}{N} \sum_{n=0}^{N-1} \xi_n^2\right) \\ &= \sigma^2 \end{aligned}$$

The agreement between the two methods of the white noise amplitude estimation allows us to take the calculated value as a good approximation.



Supplementary Figure 3. Average of  $|\mathcal{F}(\xi_n)_k|^2$  from all horizontal lines for two different AFM images. The black line represents the  $\sigma$  value we found before by analysis of variance of signal measured on a glass substrate. It is approached by the absolute value of Fourier coefficients in regime of high frequencies, where we expect very small contributions from features measured (for example cell nucleus).