The mechanical properties of cells measured using micro-fluidic devices

Application to atherosclerosis

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ABSTRACT

The mechanical properties of cells, such as their stiffness, adhesion, or time-dependent response to an applied stress, are mainly determined by their microstructure. There are many clues that show that cells in a diseased state have different mechanical properties than healthy cells due to a change in microstructure. Known examples are cancer, malaria, and leukemia. Atherosclerosis may be another disease that could be associated with mechanical properties of white blood cells, in particular of monocytes which play a key role in the development of atherosclerosis.

In this work, we study the effect of atherosclerosis on the mechanical properties of circulating monocytes for the first time, using micro-fluidic devices with narrow channels. We use two types of micro-fluidic devices: a so-called one-channel design that has just one channel that is narrower than the diameter of the monocytes, and a capillary network device, having a number of parallel narrow channels. The monocytes are flushed through the device, and are physically deformed in the narrow channels by interaction with the walls. The deformation and speed of the cells are monitored using a (high speed) camera and analyzed using dedicated image analysis software. Any change in mechanical properties (stiffness and/or adhesion) is expected to result in a change in cell velocity. We mimic a number of processes that take place during the development of atherosclerosis by treating the monocytes (THP-1 cells in our case) with Low Density Lipoprotein (LDL) and the inflammatory agent Phorbol 12-myristate 13-acetate (PMA). In addition, the channel walls are coated with the vascular adhesion molecule (VCAM-1) to influence the cell-wall interaction.

The velocities of the cells in the micro-fluidic one-channel device are successfully determined. None of the treatments mimicking processes in atherosclerosis, though, result in a significant change in cell velocities in our device, even though we observe changes in morphology, as well as changes in expression of adhesion molecules and activation markers.

We use a semi-empirical model to understand our experimental results. The agreement between the model prediction and the measured data is good. An important conclusion of the model analysis is, however, that our measurements are not sufficiently sensitive to distinguish between effective elastic moduli of cells over a range of 2 orders of magnitude (between 0.1 and 10 kPa). This means that, even though the treatments could lead to a change in mechanical properties, this may not show up in the measurements significantly, at least for our particular design and measurement conditions. The model indicates that decreasing the channel cross section, lowering the flow rate or increasing the number of parallel channels to lower effective flow rate will help to increase the sensitivity to mechanical changes. The capillary network device is therefore in principle more sensitive. Another important advantage of the device is that may enable high throughput analysis. However, the capillary network device turns out not be suitable for the reliable study of the mechanical properties of cells due to temporary or permanent blockage of parts of the network by cell clogging.
1. INTRODUCTION

In this chapter, we give a brief introduction into the mechanical properties of cells and their relationship with diseases in general. Then, we explain why the measurement of the mechanical properties of white blood cells may be useful for our application: atherosclerosis, and describe how we will use micro-fluidic devices to study these properties and link them to the disease.

Mechanical properties of cells and diseases

The mechanical properties of cells, such as their stiffness, adhesion, or time-dependent response to an applied stress, are mainly determined by their microstructure. The main components of the microstructure are the membrane, cytoplasm, cytoskeleton, nucleus, and organelles within the cytoplasm, see Figure 1. The cytoskeleton consists of an actin network, intermediate filaments and microtubules.

![Figure 1: A schematic illustration of the microstructure of a typical eukaryotic cell](image)

The specific structure, and therefore the mechanical properties, may be different for different types of cells. There are many clues that show that cells in a diseased state have different mechanical properties than healthy cells due to a change in microstructure. Known examples are cancer, malaria, and leukemia\(^\text{[1][2][3][4][5]}\). On the basis of a reliable measurement of mechanical properties of cells, in particular cell stiffness, it may therefore be possible to distinguish and select a certain type of cell, affected cells from healthy cells for a variety of diseases, and cells in a particular stage of a disease.

In the present work, we study the possible effect of atherosclerosis on the mechanical properties of monocytes, a class of circulating white blood cells, for the first time.

White blood cells

White blood cells, or leukocytes, are activated by the immune system to defend the body against both infectious diseases and foreign materials. Five main classes of leukocytes exist, each having different functions in the body. The full classification of leukocytes is given in Appendix I. Leukocytes constitute only 1% of the blood volume, of which
monocytes only represent 6%, whereas red blood cells constitute almost one half of the blood volume. However, the mechanical properties of white blood cells are important since they are about twice larger in volume per cell and stiffer than the red blood cells\textsuperscript{[6]}. Monocytes, for example, have an average diameter of 15-20 µm whereas the average size of red blood cells is 8 µm.

A number of diseases have been shown to be correlated with changes in mechanical properties of white blood cells, e.g. leukemia\textsuperscript{[5][7]}, ischemia\textsuperscript{[8][9]}, leukostasis\textsuperscript{[7]}, and retinopathy\textsuperscript{[10]}. The change in properties of the latter three is caused by a change of the activation state of the cells. The activation results in different mechanical properties due to an increased adhesion and a rearrangement and crosslinking of the actin network\textsuperscript{[11][12][13]} An overview of diseases which are associated with a change in mechanical properties of white blood cells is given in Appendix III.

\textbf{The function of monocytes}

The function of monocytes is to protect tissue from foreign substances. In response to an inflammation in the tissue, monocytes are activated, leave the bloodstream via the inner layer of the blood vessel, the endothelium, and migrate into the tissue. This process is shown in Figure 2A. After differentiation into so-called macrophages, they attack and digest foreign microorganisms and dead cell debris. Adhesion molecules are involved in the adhesion of monocytes to endothelial cells and in their migration into the subendothelial space. Monocytes come in random contact with endothelial cells, and the adhesion molecule \textit{E-selectin} expressed by endothelial cells causes a slowing down of monocytes flowing in the active blood stream. As a consequence they roll over the endothelium. Then, monocytes attach firmly with integrins\textsuperscript{1} on their surface (VLA-4 and \textit{Mac-1}) to endothelial adhesion receptors, i.e. vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1). Once monocytes are tightly bound, they migrate into the subendothelial space in response to monocyte chemotactic protein-1 (MCP-1) expressed by endothelial cells\textsuperscript{[14]}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2.png}
\caption{(A): The migration into the tissue of monocytes as an inflammatory and immune response to carry out their function; (B): The first phase of atherosclerosis development, in which Low Density Lipoprotein (LDL) interferes with the process of the migration\textsuperscript{[14]}.

1 Integrins are cell surface receptors that play a role in the attachment of cells to other cells.}
Our application: atherosclerosis and the role of monocytes

Atherosclerosis may be another disease that could be associated with mechanical properties of white blood cells, in particular of monocytes which play a key role in the development of atherosclerosis[12], since this modifies the process of transmigration of the monocytes into the tissue, as shown in Figure 2B.

Atherosclerosis is a chronic inflammatory condition which occurs within discrete regions of the arterial walls. The disease begins when the inner layer, the endothelium, is damaged. There can be various causes of this damage, such as high cholesterol level, smoking, hypertension, diabetes, and obesity[15][16]. As a result of the damage of the endothelium, its permeability for Low Density Lipoprotein (LDL)\(^2\) increases, so that this lipoprotein accumulates in the subendothelial space. Having arrived in the subendothelial space, LDL undergoes oxidation. The accumulation of oxidized LDL activates endothelial cells to express monocyte chemotactic protein 1 (MCP-1), which attracts monocytes from the blood stream into the subendothelial space. There, the monocytes differentiate into macrophages. The macrophages release a variety of chemicals, including cytokines\(^3\), in order to activate endothelial cells to express adhesion molecules, i.e. VCAM-1 and ICAM-1, which bind to integrins on monocyte surfaces, i.e. VLA-4 and Mac-1, respectively, making them available for recruitment into the subendothelial space by MCP-1. Subsequently, by taking up oxidized LDL, the macrophages slowly turn into large so called "foam cells". This loop of adhesion, migration, differentiating, adhesion molecule expression, and uptake of oxidized LDL, repeats\(^14\). This is the beginning of atherosclerotic plaque formation which continues by the accumulation of fat-laden foam cells, smooth muscle cells and other materials, to form a patchy deposit called atherosclerotic plaque, see Figure 3. As this grows, it thickens the artery wall and bulges into the artery. This may narrow or block an artery, reducing or stopping blood flow. Since the arteries supply the myocardium (the muscle of the heart) with oxygen and nutrients, this results in Coronary Artery Disease (CAD) (or atherosclerotic heart disease) i.e. a lack of oxygen supply to heart tissues (ischemia) which can lead to death of heart tissue and over time to heart failure.

\(^2\) Low Density Lipoprotein (LDL) is a lipoprotein that is present in the bloodstream at optimal levels between 100 and 129 mg/dl; its normal function is to carry fats and cholesterol from the liver to peripheral tissues.

\(^3\) Cytokines are a category of signaling molecules that, like hormones, are used extensively in cellular communication. They are proteins or glycoproteins.
Up to now, no studies have been published on the possible effect of atherosclerosis on the mechanical properties of monocytes. It could be speculated, that the activation of the monocytes by cell adhesion molecules changes their morphology and therefore their mechanical properties. Also, the presence of native LDL\(^4\) has been shown to change the expression by monocytes of a number adhesion and activation markers, which may lead to changes in their adhesive and mechanical properties, see Chapter 2.

On the other hand, the activation may not be specific for atherosclerosis, since many diseases may activate monocytes by a response of the immune system. However if we have some previous knowledge about the presence of atherosclerosis, we may differentiate between the stages of the disease through a measurement of the mechanical properties of the monocytes and make a comparison of the change of deformability.

**The measurement of mechanical cell properties**

Several techniques exist for the measurement of cellular mechanical properties. An overview is given in Appendix II. The techniques can be roughly divided into those methods that probe only part of the cell, and those that deform cells globally. The first category includes techniques such as *Atomic Force Microscopy* (AFM)\(^5\), *magnetic twisting cytometry*\(^{17}\) and *cytoindentation*\(^{11}\). A disadvantage of these approaches is that the response may depend significantly on the precise probing location, since only part of the cell is probed. As a result, these techniques generally show a large cell-to-cell spread. Techniques in which cells are deformed globally, i.e. the second category, are *laser/optical cell stretching*\(^{18}\), *microplate stretching*\(^{19}\), *microfabricated post array detection*\(^{20}\), *micropipette aspiration*\(^{21}\) and *cell compression testing*\(^{22}\).

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\(^4\) Native LDL means not oxidized LDL.
Many of these techniques are performed on a per-cell basis. Therefore, they require single-cell handling which can make them very tedious and slow (several cells per hour). For a proper diagnosis of diseases, often, statistics on a large number of cells is needed to achieve adequate sensitivity and specificity. Moreover, for some diseases it is necessary that it is diagnosed quickly, so that the proper treatment can be received by the patient in time. Furthermore, in many methods it is impossible to create a suitable environment around the cells to be tested, leading to rather artificial and irrelevant results.

Recently, micro-fluidic approaches have been used to characterize the mechanical properties of biological cells. A micro-fluidic device contains sub-millimeter channels, down to sizes of just a few micrometers, in which liquids and (bio-)molecules can be manipulated. One advantage of micro-fluidics is that it offers opportunities to study mechanical properties of a single, non-isolated cell in a capillary-like microenvironment, under physiological conditions, for example in fresh blood. Control of the cell’s micro-environment is particularly important for obtaining meaningful results using white blood cells, since the interaction between the cells and their environment determines their activation state, which substantially influences their behavior. An additional advantage of micro-fluidic devices is that high-throughput testing of cells is possible by a proper design of the micro-fluidic channel structure, so that a proper statistical analysis can be done while the testing time is limited.

Shelby et al.\cite{23} monitored the deformability and the shape recovery time of a single uninfected and malaria infected red blood cell by flushing through capillary-like channels in such a micro-fluidic chip. Rosenbluth et al.\cite{7} developed a micro-fluidic device that resembles the micro-capillary network in vivo, and characterized the properties of leukemic cells.

**Our approach: monocytes in micro-fluidic devices**

For the first time, we study the possible influence of atherosclerosis on the mechanical properties of circulating monocytes by mimicking a number of processes that take place during the development of atherosclerosis. Two types of micro-fluidic devices are used, shown in Figure 4. One device, the so-called one-channel design, has just one channel that is narrower than the diameter of the monocytes, see Figure 4A. The other device is a capillary network design, having a number of parallel narrow channels. This mimics the capillary network in vivo, see Figure 4B. Both devices have larger by-pass channels to limit the flow velocity in the narrow channels. The micro-fluidic channels are made by positioning polydimethylsiloxane (PDMS) structures on a glass substrate.
The monocytes are introduced into the device, and are physically deformed in the narrow channels by interaction with the walls. The deformation and speed of the cells are monitored using a (high speed) camera. Any change in mechanical properties (stiffness and/or adhesion) is expected to result in a change in cell velocity.

The possible influence of atherosclerosis is studied by cell and/or device treatments that mimic processes taking place during the development of atherosclerosis. In particular, the effect of a high cholesterol level in the blood was examined by a high native Low Density Lipoprotein (LDL) treatment of monocytes. Also, the effect of the vascular adhesion molecule-1 (VCAM-1) on native LDL-treated cells was studied by coating the channel walls with VCAM-1. Finally, the process of differentiation of monocytes into macrophages, and the possible corresponding change in mechanical properties, was studied by a treatment of the cells with an inflammatory agent, Phorbol 12-myristate 13-acetate (PMA), which stimulates their differentiation into macrophages.

**Objective of our work**

The overall objective of this work is to use the micro-fluidic devices to study the effect of atherosclerosis on the mechanical properties of circulating monocytes by mimicking a number of processes that take place during the development of atherosclerosis. More specifically, our aim is to establish whether any effect in cell velocity occurs and if this can be detected using the micro-fluidic devices.
Structure of the report

Chapter 2 investigates the effects of the cell treatments that mimic processes taking place during atherosclerosis development, on the expression of selected adhesion and activation markers of monocytes. Chapter 3 contains a brief overview of models that have been used to describe the mechanical properties of cells. In more detail, a semi-empirical model is explained that has been used to describe the behavior of cells flowing through capillaries, and which is also used later on to explain our own measurements. Chapter 4 deals with the one-channel design, which is described and analyzed with a flow analysis model. This chapter also contains the measurement results which are explained by applying the semi-empirical model. In Chapter 5, the same is done for the capillary network device (model analysis, measurement results, and experimental analysis). In Chapter 6, all the experimental details of our work are described, including the cell culture and treatment procedure, the manufacturing of the devices, the experimental set-up and procedures, and the image analysis. The basics have also been included in Chapters 4 and 5, so that these can be understood without referring to Chapter 6 which may be consulted by a reader who wishes to know more details. Finally, Chapters 7 and 8 end the report with conclusions and recommendations.

The appendices contain information about topics relevant to our work: a classification of circulating cells, existing techniques for measuring mechanical properties of cells, diseases associated with a change in mechanical properties of cells, and the developed image analysis code.
2. BIOLOGICAL EFFECTS OF CELL TREATMENTS RELATED TO ATHEROSCLEROSIS

This chapter investigates the effects of the cell treatments that mimic processes taking place during atherosclerosis development, on the expression of selected adhesion and activation markers of monocytes. Such measurements are essential to prove that the treatments have an effect. A positive test result will provide the motivation for further investigation of the mechanical property changes of the cells.

Treatments

Cells from a human acute monocytic leukemia cell line\(^5\), THP-1 cells, were used in this study. It should be noted that while THP-1 cells are called monocytes, they are actually more representative of monoblasts, a slightly immature form of monocytes (see Appendix I for a classification of blood cells). A number of treatments were applied to the cells.

First, the effect of a high cholesterol level in the blood was examined by a high Low Density Lipoprotein (LDL) treatment of monocytes. LDL is a type of lipoprotein that delivers fats and cholesterol within the water based solution of the blood stream from the liver to peripheral tissues. Its optimal blood level is between 100 and 129 mg/dl (i.e. 1000-1290 µg/mL).

LDL plays multiple roles in atherosclerosis.

- As mentioned in Chapter 1, native LDL migrates from the blood stream through the endothelium and undergoes oxidation in the subendothelial space. This attracts monocytes from the blood stream into the subendothelial space where they differentiate into macrophages.
- In the subendothelial space, the oxidized LDL is taken up by the macrophages, which causes them to differentiate into foam cells.
- Also the level of native LDL in the blood stream (rather than oxidized LDL present in the subendothelial space) has been shown to affect the process of monocyte recruitment into the subendothelial space. Han et al.\(^{[24]}\), namely, have shown that an increased level of native LDL which is characteristic for high cholesterol level causes an increased integrin expression on the surfaces of THP-1 cells and an enhanced chemotactic response of the cells to the chemoattractant protein-1 (MCP-1), see Figure 2. This increases the adhesion of monocytes to endothelial cells\(^{[24][25]}\) and leads to an increased monocyte accumulation in the vessel wall during atherosclerosis.

\(^5\) Cells isolated directly from a person (known as primary cells) have a limited lifespan. After a certain number of cell divisions the cells stop dividing, while retaining viability. Therefore, in our experiments a human acute monocytic leukemia cell line, THP-1, was used that was isolated from a leukemia patient. This cell line divides indefinitely while their properties are maintained.
Second, the process of differentiation of monocytes into macrophages was studied by a treatment of the cells with an inflammatory agent, Phorbol 12-myristate 13-acetate (PMA). The PMA treatment differentiates THP-1 cells into cells with the morphological and functional characteristics of mature activated macrophages in vitro, i.e. it up regulates CD14 expression levels, which is a surface marker of macrophages. Differentiated THP-1 macrophages have been widely used as an in-vitro model of human macrophage involvement in an inflammatory disease[26].

The procedure of the treatments is described in section 6.1.

**Experimental results**

We studied the change of selected adhesion and activation markers on THP-1 cells due to a treatment with 20 and 100 µg/mL LDL and with 4 nM PMA. Table 1 shows the list of the selected markers, and their localization, ligands and function.

<table>
<thead>
<tr>
<th>Alternative Designation</th>
<th>Localization</th>
<th>Ligand</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td>CD9</td>
<td>Cell surface glycoprotein</td>
<td>All leukocytes</td>
<td>Multiple functions/ligands</td>
</tr>
<tr>
<td>CD11a</td>
<td>LFA-1</td>
<td>All leukocytes</td>
<td>ICAM-1, ICAM-2</td>
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<tr>
<td>CD11b</td>
<td>Mac-1</td>
<td>Granulocytes, monocytes</td>
<td>ICAM-1</td>
</tr>
<tr>
<td>CD31</td>
<td>PECAM-1</td>
<td>Endothelium, leukocytes, platelets</td>
<td>PECAM-1</td>
</tr>
<tr>
<td>CD49d</td>
<td>VLA-4</td>
<td>Lymphocytes, eosinophils, basophils, monocytes</td>
<td>VCAM-1</td>
</tr>
<tr>
<td>CD54</td>
<td>ICAM-1</td>
<td>Endothelium, monocytes</td>
<td>CD11a</td>
</tr>
<tr>
<td>CD62L</td>
<td>L-Selectin</td>
<td>All leukocytes</td>
<td>P-E-selectin, GlyCAM-1, CD14, MAdCAM-1</td>
</tr>
<tr>
<td>CD162</td>
<td>P-selectin glycoprotein ligand-1</td>
<td>All leukocytes</td>
<td>P-E-, L-selectin</td>
</tr>
<tr>
<td>HLA-DR</td>
<td>Cell surface receptor</td>
<td>Macrophages, B-lymphocytes, dendritic cells</td>
<td>Foreign peptide antigen</td>
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*LFA: lymphocyte function associated antigen; VLA: very late antigen; CAM: cell adhesion molecule; ICAM: intercellular CAM; MAdCAM: mucosal addressin CAM; PECAM: platelet endothelial CAM; VCAM: vascular endothelial CAM; MAdCAM: mucosal addressin CAM; GlyCAM: Glycosylation dependent CAM

6 A ligand is a substance that is able to bind to and form a complex with a biomolecule to serve a biological purpose.
The measurement of the change in expression of these selected markers was performed as follows: Fluorescently labeled antibodies are used to stain untreated and treated cells for specific protein targets present on their surface. By using Fluorescence Activated Cell Sorting (FACS), which sends a laser beam onto single cells to excite the fluorophores bound to selected antibodies, a fluorescence intensity signal is captured that is proportional to the amount of fluorescent probe (i.e. antibody) bound to a single cell. The untreated cells act as a reference. If the fluorescence intensity goes up with a treatment, then it means that there is more of a specific protein present on the cell surface (protein up regulation, increased expression). If the fluorescence intensity goes down, then it means that there is less of a specific protein present on the cell (protein down regulation, decreased expression).

The results are shown in Figure 5 which shows the fold change, i.e. the relative change compared to untreated cells, in expression of the selected adhesion and activation markers on THP-1 cells treated with 20 µg/mL LDL. It can be concluded, that the 20 µg/mL LDL treated THP-1 cells do not show a remarkable change in expression of selected adhesion and activation markers compared to untreated cells.

The results for the THP-1 cells treated with 100 µg/mL LDL and 4 nM PMA are shown in Figure 6. Indeed, compared to the untreated cells the 100 µg/mL LDL treated THP-1 cells do show an increase in expression of CD9, CD11a, CD31, CD49d, CD54, CD162 and a decrease of CD14, CD62L and HLA-DR. THP-1 cells treated with 4 nM PMA do show an increase of CD9, CD11a, CD11b, CD14, CD31, CD54, CD62L, HLA-DR and a decrease of CD49d and CD162. Especially, the change for the PMA-treated cells is substantial.
Figure 6: Change in expression of selected adhesion and activation markers on untreated THP-1 cells and THP-1 cells treated with 100 µg/mL LDL and 4 nM PMA for 24 hours. The fold change of “1” means that there is no change in terms of expression or activation. (measurements by Dr. D. Merkle)

Figure 7 shows images of THP-1 cells treated with 15 µg/mL fluorescent LDL. It can be concluded that the LDL is taken up heterogeneously by the cells.

Figure 7: Images of THP-1 cells with a 15 µg/mL fluorescent LDL treatment. A: Taken by a conventional optical microscope; B: Taken by a fluorescent microscope. C: The grey scale image of A is combined with the image of B.
Conclusion

- A change in expression of selected adhesion and activation markers on the 100 ug/mL LDL and 4 nM PMA treated THP-1 cells is found. Therefore, we may speculate that there may also be mechanical changes due to these treatments. The 20 ug/mL LDL treatment, on the other hand, did not show any significant expression change, and therefore the mechanical properties may be unaffected.
- The uptake of LDL is heterogeneous, and therefore the LDL treatment may lead to cell-to-cell variations of the properties.
- The 100 ug/mL LDL treatment increases the expression of the integrin VLA-4 on the THP-1 surfaces as is evidenced by the increase of CD49d expression in Figure 6. In vivo, VLA-4 binds to vascular adhesion molecule-1 (VCAM-1) which is expressed by the endothelial cells. It is expected, therefore, that the adhesion of THP-1 cells to VCAM-1 is increased by the presence of native LDL. Therefore, we will study the effect of coating the channel walls with VCAM-1 on the behavior of the 100 ug/mL LDL treated cells in our devices.
3. THEORY: MODELS FOR CELLS FLOWING THROUGH CAPILLARIES

In this chapter, the existing models for mechanical properties of white blood cells are briefly described. Then, the approaches that have been used to model the flow of cells through capillaries are explained. These range from semi-empirical descriptions based on dimensional analysis, to analytical models to numerical models. Later in this report, one of the semi-empirical models is used to explain the experimental results.

Mechanical models for white blood cells

The mechanical properties of white blood cells have been modeled with various levels of complexity. AFM and especially micropipette aspiration experiments, described in Appendix II, have been widely used to fit the mechanical models proposed in the literature, and extract the model parameters. A detailed overview of the models is given in Yavuz et al.\[28\].

Two main classifications exist for mechanical models used for passive white blood cells\(^7\). The first class of mechanical models does not evaluate the contributions of the cell membrane and the complex cell interior separately by modeling the cell as a homogeneous solid sphere. The simplest approach is to simply assume a linear elastic behavior, using an effective elastic modulus to describe the cell’s behavior. This was done by Rosenbluth et al.\[^5\] who found values between 10 and 1000 Pa for white blood cells. The standard linear viscoelastic solid model represents the white blood cell with a Maxwell element (an elastic and a viscous element in series) parallel with an elastic “Hookean” element. This is the simplest model capable of accounting for the overall behavior of immediate elastic response, viscoelastic creep and a final equilibrium configuration of a passive leukocyte\[^29\].

The second class of mechanical models makes a distinction between the cell’s surface membrane (the cortical shell) and the complex interior part of the cell (the liquid core), i.e. the cortical shell - liquid core models. Depending on the specific model, the cortical shell may represent surface tension, viscous effects and/or elastic properties. The liquid core is a homogeneous representation of the cell’s complex interior which consists of the cytoplasm, the nucleus, organelles and several granules.

The Newtonian liquid drop model\[^30\][\[^31\]) is the simplest cortical shell-liquid core model and describes the cell surface with a constant surface tension (with a typical value of \(35 \cdot 10^{-4} \text{N/m}\)) and the cell interior with a Newtonian fluid\[^8\] (with a typical viscosity of \(210 \pm 100 \text{Pa.s}\)). It has been observed that the apparent viscosity of the cell interior may depend on deformation and deformation rate. To represent this dependency, the shear

\[^7\] The effect of activation is often modeled with a change of effective model parameters.
\[^8\] For a Newtonian fluid, the stress is proportional to strain rate, with the viscosity being the proportionality constant.
A *thinning liquid core model* expresses the liquid core of the cell as a power-law fluid whereas the cortical shell is still modeled with a constant surface tension. This model can describe the rheological behavior of a cell at large deformations[32].

However, these two cortical shell-liquid core models cannot reproduce some features observed in the micropipette experiments, such as the initial elastic response, when a stress is suddenly applied, or the initial elastic recoil on sudden release of stress, since the core is assumed to be a viscous fluid, and the shell does not have any elastic properties.

One extension to this is to model the shell as an elastic membrane, with an effective elastic modulus, filled with an incompressible viscous liquid, as has been done in the semi-empirical models of Lee & Fung[33] and Hochmuth[34] (this is the model we will use to analyze our results, see the next section). Another extension is the *Maxwell liquid drop model*[35] which represents the cell membrane as a cortical shell with a constant surface tension and the heterogeneous cell interior as a viscoelastic Maxwell fluid. The model can explain the cell behavior both during small deformations of leukocytes and also during recovery phase following a large deformation. The *Extended Maxwell liquid drop model*[36] represents the cortical shell with elastic effects, i.e. elastic stretching and shearing, in addition to a constant surface tension.

Modeling the interior of white blood cell as a liquid-like body neglects the complex internal structures that include nuclei, granules, and other organellar bodies. The *Newtonian compound drop model*[37] is the first model that takes the nucleus into account. The model represents the nucleus by a Newtonian fluid core region with a high viscosity and the cytoplasm by a thick layer of Newtonian fluid with low viscosity, and the surface membrane by a cortical shell with a constant surface tension. The core region and the cytoplasm layer are separated by a shell of constant surface tension.

One big step beyond the Newtonian compound drop model is to represent the leukocyte by a *Maxwell compound drop model* with one core region or a *Newtonian compound drop model with multicore regions*. The latter model is the proposed model for modeling the nucleus of leukocytes with multilobulated shape.

**Cells through capillaries and channels: dimensional analysis**

The capillaries are the smallest blood vessels in the circulatory system with diameters typically ranging from 4 to 10 μm. They are the primary sites where an exchange of nutrients with the interstitial liquid in the tissues takes place. The walls consist of a single layer of endothelial cells lying on a basement membrane. Capillary blood flow, so called microcirculation, does not behave as a homogeneous flow since the diameter of the capillaries is smaller than the blood cells. They must deform in order to pass the capillaries while moving in a single file. The Reynolds number for plasma in capillaries is very low (10^-3 - 10^-2) so that the inertial forces can be ignored.

In our devices, we mimic the deformation of cells passing through capillaries with the use of micro-fluidic devices. A main difference between the device and real capillaries is that
the former has a rectangular cross section, whereas the latter are circular. In this chapter, all models are based on circular channels and we will make the translation to our geometry in section 4.3. Other differences are the chemical interactions with the cells and the mechanical properties of the walls. We will address these issues later as well.

A first understanding of what determines the cell velocity both in capillaries and in our devices can be obtained by carrying out a basic dimensional analysis (see also Lee & Fung [33]). The quantities that are relevant for the analysis are sketched in Figure 8. In the analysis, we make a number of assumptions and simplifications as follows:

(i) The cells are assumed to be spherical when undeformed (as is the case for monocytes).
(ii) It is assumed that, mechanically, the cell can be represented with an elastic membrane with an effective elastic modulus \( E_c \), filled with an incompressible viscous liquid with a representative viscosity \( \mu_c \). As mentioned in the previous section, this is a very primitive approximation of the reality which neglects, for example, the fact that the cell is heterogeneous, and that surface tension can play a role [28]. For a first basic dimensional analysis, however, this gives already insight.
(iii)The cells are assumed to be spaced far from each other so that there is no cell-to-cell interaction.
(iv) We assume that the capillary wall is much stiffer than the cell so that the elastic properties of the capillary or the channel can be neglected since this is certainly the case for the devices we use (i.e. the PDMS we use for our channels has an elastic modulus of around 1 MPA which is much higher than that of the cells which is expected to be between 0.1 and 1 kPa, see Figure 76).

\[
\begin{align*}
\text{Figure 8: The sketch of the quantities that are relevant for basic dimensional analysis of cells flowing through the capillaries.} \\
&\text{The cell velocity is} \ V_c, \ \text{and the average velocity of the fluid flow (i.e. the plasma in the blood vessels)} \ \text{is} \ V_m. \ \text{The typical dimensions are the diameter of the undeformed cell} \ D_c, \ \text{the cell membrane thickness} \ h_c, \ \text{the diameter of the capillary} \ D_T, \ \text{which represents in our case the dimension of the cross-section of the channel, and the capillary length} \ L_T. \ \text{The viscosity and density of the fluid (or plasma) are} \ \mu \ \text{and} \ \rho, \ \text{respectively. The cell position in the capillary is} \ X. \ \text{Finally, the interaction or adhesion between the cell and the capillary wall, which we will express as a friction coefficient} \ f_{adh}, \ \text{can affect the cell behavior.}
\end{align*}
\]

To obtain the dimensionless numbers relevant for the problem, we should formally analyze the governing equations, which are the momentum equation for the fluid flow,
the force balance over cell, and the constitutive equations for the cell and the fluid, in particular by scaling these equations with characteristic parameters. However we will derive the dimensionless numbers on a phenomenological approach. We can categorize the dimensionless numbers into four classes.

- **Geometry-related:**
  - \( \frac{L_x}{D_T} \) and \( \frac{X}{L_T} \) characterize the channel geometry,
  - \( \frac{h_c}{D_c} \) is the relative membrane thickness,
  - \( \frac{D_c}{D_T} \) characterizes the cell deformation.

- **Flow-related:**
  - \( \frac{\rho V_m D_T}{\mu} \) is the Reynolds number characterizing the fluid flow.

- **Forces on cell – related:**
  The force balance on the cell is determined by the viscous stress applied by the flow characterized by \( \mu \frac{V}{D_T} \), the elastic stress in the cell characterized by \( E_c \frac{D_c}{D_T} \), and the cell’s internal viscous stress characterized by \( \mu_c \frac{V}{D_c} \). The ratios of these three lead to two independent dimensionless numbers, namely
    - \( \frac{\mu V_c}{E_c D_c} \), representing the ratio of the viscous stress acting on the cell over the elastic stress, and
    - \( \frac{\mu_c}{\mu} \), representing the cell’s internal viscous stress over the viscous flow stress.
    - In addition, also the direct adhesion force between the cell and the channel wall is relevant, and is represented by the friction coefficient \( f_{adh} \).

- **Finally, since the cell is viscoelastic, the ratio of time scale of the convective flow to the characteristic time scale of the cell may also play a role. This is characterized by\(^9\)**
  - \( \frac{L_T}{V_m} \)
  - \( \frac{\mu_c}{E_c} \)

In total, we have found the following nine following dimensionless parameters:

\(^9\) This dimensionless number is the so-called “Deborah number” of the problem.
As we will show later, the Reynolds number \( \frac{\rho V_m D_T}{\mu} \) is much smaller than 1 both in our experiments and in capillaries, hence it is not important. If the channel or capillary length is much larger than its diameter, \( \frac{L_T}{D_T} \gg 1 \), and the cell position is not close to the ends, then \( \frac{L_T}{D_T} \) and \( \frac{X}{L_T} \) are also not playing a significant role. With the assumptions mentioned earlier, the cell velocity, non-dimensionalized with the fluid velocity can be expressed using the following functional relationship:

\[
\frac{V_c}{V_m} = f\left(\frac{D_c}{D_T}, \frac{h_c}{D_c}, \frac{\mu V_c}{E_c D_c}, \frac{L_T}{V_m}, \frac{\mu_c}{E_c}, \mu_c, f_{adh}\right)
\]

Equation 1

The presence of the cell in the capillary will change the flow resistance, and therefore it will affect the relationship between the applied pressure drop and the flow rate in the capillary. For any channel flow, the relationship between the applied pressure drop \( \Delta p \) and flow rate \( Q \) is:

\[
\Delta p = R_H Q
\]

Equation 2

In which \( R_H \) is the hydrodynamic resistance. For a cylindrical Poiseuille flow (when no cell is present), this is given by:

\[
R_H = \frac{128 \mu L_T}{\pi D_T^4}
\]

Equation 3

Substituting Equation 3 into Equation 2, and expressing the flow rate in terms of the mean velocity ( \( Q = \pi V_m D_T^2 / 4 \) ), we get:

\[
\Delta p = \frac{32 \mu V_m L_T}{D_T}
\]

Equation 4

The presence of the cell will introduce an additional pressure drop which is determined by the same dimensionless numbers as in Equation 1. To be consistent with Equation 4 with respect to the dimensions, the additional pressure drop can be written as:

\[
\Delta p_c = \frac{32 \mu V_c}{D_T} F\left(\frac{D_c}{D_T}, \frac{h_c}{D_c}, \frac{\mu V_c}{E_c D_c}, \frac{L_T}{V_m}, \frac{\mu_c}{E_c}, \mu, f_{adh}\right)
\]

Equation 5
In which $F$ is a non-dimensional function. The total pressure drop is the sum of Equation 4 and Equation 5. Rewriting the sum of equations in the form of Equation 2, we get:

$$
\Delta p = R_m \left( 1 + \frac{D_T}{L_T} \frac{V_c}{V_m} F \right) Q
$$

Equation 6

In which $F$ depends on the dimensionless numbers $\frac{D_c}{D_T}$, $\frac{h_c}{D_c}$, $\frac{\mu V_c}{E_c D_c}$, $\frac{L_T}{V_m}$, $\frac{\mu_c}{\mu}$, and $f_{adh}$. In conclusion, the presence of the cell will lead to an increased hydrodynamic resistance by a factor of $\left( 1 + \frac{D_T}{L_T} \frac{V_c}{V_m} F \right)$.

The functions $f$ and $F$ in Equation 1 and Equation 6, respectively, have been experimentally studied by Lee & Fung$^{[33]}$ and Hochmuth$^{[34]}$. We will summarize these studies in the next section. Later in section 4.3, these semi-empirical models will be used to analyze our experimental data.

**Cells through capillaries and channels: empirical models**

Lee & Fung$^{[33]}$ have performed large-scale model experiments and a semi-empirical analysis of single cells flowing through capillary vessels. The model cells were relatively large, hollow thin-walled rubber cells, having the bi-concave shape of red blood cells, filled with a liquid. The diameter of the rubber cells was 4.29 cm, the wall thickness was 0.042±0.01 cm, i.e. the ratio between wall thickness and diameter was of the order 0.01. The rubber material had an elastic modulus of 250 kPa and the interior fluid was silicone oil with a viscosity of 29.5 Pa·s. These properties were mentioned to be representative for highly strained red blood cells. The rubber cells were pumped through various tubes, with three different diameters, so that the ratio of sphere to tube diameter $\frac{D_c}{D_T}$ was varied between 1.36, 1.13, and 0.98 (similar to what can be expected in the microcirculation). The mean flow velocities of the carrying fluid, which was the same silicone fluid as the filling fluid, was varied between $V_m=0.03$ cm/s and 3 cm/s. Thus, the Reynolds number was in the range $4 \cdot 10^{-4}$ to $4 \cdot 10^{-2}$, similar to values in the microcirculation. While the mean velocity was controlled, the pressure, velocity of the cells, and deformation of the cells, were measured.

The measured cell velocity was found to be linearly proportional to the applied fluid mean velocity, as can be seen in Figure 9. The data were fitted with the following empirical formula:

$$
V_c = k(V_m - \alpha)
$$

Equation 7
The fitting parameters $k$ and $\alpha$ are shown in Table 2 and they depend on the ratio of cell to tube dimensions, $\frac{D_c}{D_T}$.

![Figure 9: Measurement by Lee & Fung\[33\]: Measured cell velocity as a function of the mean flow velocity for various cell dimension / tube ratios.](image)

The fits are very good over the whole experimental range. $k$ increases over 1 as the cell/tube diameter ratio decreases. That is, smaller cells move faster than the mean velocity, so there should be “leakback” flow along the cell, which means that there must be a substantial lubrication film. Only for the largest cells measured, the cell velocity exactly equals the mean flow velocity.

**Table 2: Fit parameters in Equation 7, determined by Lee & Fung\[33\].**

<table>
<thead>
<tr>
<th>Cell/Tube diam. ratio</th>
<th>$k$</th>
<th>$\alpha$ (cm/sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.69</td>
<td>1.00</td>
<td>0.</td>
</tr>
<tr>
<td>1.36</td>
<td>1.10</td>
<td>0.02</td>
</tr>
<tr>
<td>1.13</td>
<td>1.17</td>
<td>0.10</td>
</tr>
<tr>
<td>0.98</td>
<td>1.26</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Lee & Fung\[33\] analyzed their data further using a dimensional analysis similar to that explained in the previous section. However, they did some further assumptions. First, they assumed that the time dependency of the cell, i.e. the viscosity of the cell did not play a role (since it was equal to the viscosity of the carrying liquid). This means that in Equation 6 for the pressure drop, the dependency of the function $F$ on the parameters
\( \frac{\mu_c}{\mu \mu_c / E_c} \) and \( \frac{L_T}{V_m} \) is neglected. The latter is justified if it is small since then the time scale of the convective flow is much smaller than the characteristic time scale of the cell and time-dependent behavior of the cell does not occur. Indeed, no time dependent effects were observed in the experiments of Lee & Fung. Second, direct interactions between the cell and the wall were neglected. Implicitly, Lee & Fung assumed that the cell was separated from the wall by a thin liquid lubrication layer all the time (see next section for lubrication theory). Finally, their relative membrane thickness was not varied, and therefore they were not able to study the influence of \( \frac{h_c}{D_c} \). Hence, they used Equation 6, but with a different resistance function instead of \( F \):

\[
\Delta p = R_h \left( 1 + \frac{D_c}{L_T} \frac{V_c}{V_m} f_3 \right) Q
\]

Equation 8

where \( f_3 \) depends only on the dimensionless parameters \( \frac{D_c}{D_T} \) and \( \frac{\mu V_c}{E_c D_c} \). \( f_3 \) was found by fitting the data, and the results are shown in Figure 10.

Figure 10: The non-dimensional resistance function \( f_3 \) as a function of \( \frac{\mu V_c}{E_c D_c} \) and for various \( \frac{D_c}{D_T} \), found from the measurements of Lee & Fung\(^{33}\).

Some interesting conclusions can be drawn from Figure 10. In the first place, the resistance goes up with increasing cell size, which can be expected. Second, the resistance goes down with the parameter \( \frac{\mu V_c}{E_c D_c} \) (representing the viscous stress on the
cell with respect to the elastic stress), however the effect is only substantial for large cells, and/or small $\frac{\mu V_c}{E_c D_c}$ (hence large elastic modulus $E_c$).

Sutera et al.$^{[34]}$ have done similar experiments to Lee & Fung$^{[33]}$: they also used large hollow bi-concave-shaped rubber cells flowing through tubes, and observed velocities and pressure drops. There are some main differences with Lee & Fung: the wall thickness to diameter ratio was smaller, namely order 0.005 instead of 0.01; also, the viscosity of the liquid inside the droplets was different from that of the carrier fluid. More particularly, the cells were rubber cells with a modulus of 1660 kPa and a diameter of 2 to 4 cm. The cell-to-tube diameter $\frac{D_c}{D_T}$ was varied between 1.3 and 2. The fluid in the cells had a viscosity of 12 Pa·s; that of the carrier fluid was 60 Pa·s. The Reynolds number was between $8.2 \cdot 10^{-5}$ and $1.65 \cdot 10^{-3}$, corresponding to an average fluid velocity between 0.025 and 0.5 cm/s. Figure 11 shows images of the deformation of the model cells of Sutera et al.$^{[38]}$ flowing through the tube.

![Figure 11: Images of the rubber-membrane model cells of Sutera et al.$^{[38]}$, flowing through the tube (two perpendicular views).](image)

The data were analyzed using the same approach as Lee & Fung. Figure 12 shows measured ratio of cell velocity to mean flow velocity as a function of strain parameter. The latter is defined as $\frac{\mu V_m}{E_c D_T}$, and can be transformed in a straightforward way to the similar dimensionless parameter $\frac{\mu V_c}{E_c D_c}$ used earlier. The figure contains a comparison with the data of Lee & Fung; there is a qualitative agreement. Cells with a diameter much larger than the tube diameter move with a velocity close to the mean flow velocity, however cells with a smaller diameter move faster than the mean velocity. For a fixed $\frac{D_c}{D_T}$ ratio, the parameter $\frac{\mu V_m}{E_c D_T}$ hardly affects the cell to flow velocity ratio, except for
larger $\frac{D_c}{D_r}$ for the measurements of Sutera et al. The latter suggests that the cell’s elastic modulus has a minor influence on cell velocity within the used range of measurement conditions.

The relatively small quantitative differences between the data of Sutera et al. and Lee & Fung may be caused by the differences in experimental conditions between the experiments: as mentioned earlier, the viscosity of the internal cell liquid is different, and the cell wall thickness differs. The precise reason is however not completely clear.

Figure 12: Ratio of cell velocity to mean flow velocity, as a function of strain parameter, for various cell sizes; data of Sutera et al. $^{[34]}$. A comparison is made with Lee & Fung $^{[33]}$.

Figure 13 plots the additional pressure drop, caused by the presence of a cell, on top of the pressure drop for a Poiseuille flow without a cell present. The results of Lee & Fung shown in Figure 10 can be translated in the same format, as shown in Figure 13.
Figure 13: Additional pressure drop due to the presence of a cell as a function of strain parameter, for various cell sizes; data of Sutera et al.\[34\]. A comparison is made with Lee & Fung\[33\].

There is a strong dependency on $\frac{D_c}{D_T}$ of the additional pressure drop: larger cells give higher pressure drops as expected. The strain parameter $\frac{\mu V_m}{E_c D_T}$ affects the additional pressure drop for larger $\frac{D_c}{D_T}$, but much less for the smaller $\frac{D_c}{D_T}$. The data of Lee & Fung\[33\] show the same qualitative trends, however the precise values are different. This may be explained by differences in experimental conditions as mentioned earlier.

We will apply the analysis and data of Lee & Fung\[33\] to our own measurement results in section 4.3, in order to explain our data. We did not use the data of Sutera et al.\[34\] since their ratio of carrying fluid viscosity to the interior cell viscosity is larger than one, which is opposite to the ratio in-vivo where it is smaller than one.

Cells through capillaries and channels: lubrication theory: analytical and numerical models

Lighthill\[39\] studied the problem of “elastic pellets” (which can be thought of as representing cells) that are being forced by a pressure difference to move through a tube filled with a viscous liquid. At the basis of the approach is the assumption that there is always a thin film of liquid present between the pellet and the tube wall, that is, a lubrication film exists. The relatively large pressures developed in the lubrication region will distort the cell membrane.

Lighthill solved the problem analytically. The basic approach is that three equations are solved: (i) the equation describing the flow in the lubrication layer; (ii) an equation that
solves for the lubrication layer thickness and that represents the balance between the local elastic stresses in the cell and the local pressure acting on the cell wall; here it is assumed that the undeformed cell shape is parabolic and that the behavior is linearly elastic; (iii) the global force balance between the viscous stress acting on the cell and the driving pressure. The final solutions by Lighthill are expressed in terms of dimensionless number. The form of the solutions, however, is not practical: the influence of a change in elastic modulus on a change in cell velocity, for example, cannot be determined independently.

Fitz-Gerald\textsuperscript{[40]} applied Lighthill’s theory, in axi-symmetric form, to erythrocyte motion through capillaries. As mentioned by Fitz-Gerald, the results are merely qualitative because of the crude model approximations (membrane compliance, lubrication film thickness).

More quantitative solutions were obtained by Secomb & Hsu\textsuperscript{[41]} who, much later, numerically tackled the problem. The model was axisymmetric. Lubrication theory, based on the axisymmetric model of Fitz-Gerald\textsuperscript{[40]}, was used to describe the flow in the gap between the cell and the wall. The deformability of the wall was neglected. The cell was modeled as a membrane with viscoelastic properties and an incompressible interior. A Kelvin solid model was used for the membrane, and it was allowed to deform without change in surface area. The bending stiffness of the membrane and the interior viscosity were both neglected.

In the end, three equations describe the problem: (i) the constitutive model for the membrane; (ii) local force equilibrium on the membrane; (iii) the time-dependent lubrication equation. These equations were discretized using finite differences, and solved numerically using appropriate boundary conditions (i.e. applied pressure drop or applied flow rate). The transient flow of red blood cells through short pores was simulated.

The undeformed cell was modeled as a half sphere with largest dimension of 6.6 µm. Three capillary diameters were used: 3.6, 5 and 6.3 µm. A thin shell model was used for the membrane. The membrane viscosity was set to 0.001 mN·s/m, and the membrane elastic modulus was 0.006 mN/m. The viscosity of the carrying fluid was 0.001 Pa·s. The driving pressure was varied between 10 and 100 Pa and the effect of the elastic modulus was examined by considering the effect of doubling this parameter.

In Figure 14, left, the computed change in shape of the cell while transiting through the 3.6 µm pores is depicted. Note, that the pores are relatively short, so that the computed transit time will be substantially influenced by the entry phase of the cell into the pore. The right picture shows the transit times in pores with various diameters. Transit time can be translated to cell velocity by considering the length of the pore, which is 11 µm; typical velocities are then between 11 mm/s (for 1 ms transit time) and 0.11 mm/s (for 100 ms transit time). As expected, narrower pores lead to longer transit times (smaller velocities). For higher driving pressures, the transit time goes down.
The effect of a change in mechanical properties of the cell is shown in Figure 15. The effect of membrane elasticity on transit time was strongly dependent on both the size of the pore and the driving pressure. Transit time in 6.3 µm pores was virtually unchanged by doubling membrane elasticity over the range of pressures considered here. A slight increase was seen in 5 µm pores, but only at low driving pressure. In 3.6 µm pores, transit time showed a marked sensitivity to membrane elasticity as driving pressure decreased. For pressures > 100 Pa, however, the model predicted that transit time was insensitive to membrane elasticity, even in 3.6 µm pores.
Conclusions

In Chapter 4, we will apply the semi-empirical model of Lee & Fung\cite{33} to explain the measurement results of the one-channel device. This model is based on a description of the cell using an elastic membrane and an incompressible viscous interior, and on a semi-empirical model of these cells flowing through capillaries that was constructed on the basis of experiments with hollow liquid-filled rubber model cells. Although this model is rather course, as is clear from the descriptions in this chapter, we will see in Chapter 4 that it describes the measurement results quite well.
4. ONE-CHANNEL DEVICE

In this chapter, we describe the one-channel device, and present an analysis of the expected flow within the device with the use of an analytical and a numerical model. Then, the experimental results are presented. In these experiments, treatments of the cells and the device have been used to mimic processes taking place in atherosclerosis, with the aim to determine the effect of the treatments on the mechanical behavior of the cells in the device. The results are analyzed extensively using the semi-empirical model described in Chapter 3.

The design

Our first design to characterize the mechanical properties of cells consists of a single, narrow channel, surrounded by larger by-pass channels, see Figure 16. The flow thus trifurcates which enables to reach lower flow velocities in the narrow channel at higher applied flow rates through the inlet. Also, the by-pass channels avoid a large pressure drop over the device, which in practice helps to prevent delamination of the device.

Figure 16: Design of the one-channel device. The dimensions are given in the text.

The width of the inlet channel is 200 µm and the by-pass channels have a width of 150 µm. After the trifurcation of the inlet channel, the central channel narrows in two gradual steps from 200 to 40 µm to 10 µm, and broadens in a symmetric manner. The contractions have an angle of 10˚ to the central axis. This small angle was chosen to have a gradual change of the flow to prevent the cells from clogging the contractions. The 200 µm part has a length of 500 µm; the 40 µm part has a length of 500 µm as well. The narrow channel of 10 µm width has a total length of 250 µm. The height of all channels was designed to be 15 µm. The actual precise width and height were influenced by subtle details in the processing. Using confocal microscopy, the actual width and height of the narrow channel was measured to be 11.5 µm and 14 µm, respectively (see section 6.2).

The device may be used to characterize the mechanical properties of cells. The hypothesis is that stiffer cells, being flushed through the narrow channel, travel more slowly than cells that are more compliant. Hence, by a measurement of the transit times of cells through the narrow channel, we may be able to distinguish between stiff and compliant cells.
4.1 FLOW ANALYSIS OF THE DESIGN

Resistance analogue model

A resistance analogue model is used to analyze the flow behavior of the one-channel design. This model uses the principle that, for each of the channel sections in the device, the flow rate \( Q \) [m\(^3\)/s or \( \mu \text{L/min} \)] is proportional to the pressure drop over the section, \( p_{\text{in}} - p_{\text{out}} \) [Pa], and the proportionality constant is the hydraulic resistance \( R_{\text{hyd}} \) [Pa·s/m\(^3\)]:

\[
p_{\text{in}} - p_{\text{out}} = R_{\text{hyd}} Q
\]

Equation 9

For a single rectangular channel, with length \( L \), height \( h \), and width \( w \), and for a fluid with dynamic viscosity \( \mu \), the hydraulic resistance is given by (see Bruus\(^{42}\)):

\[
R_{\text{hyd}} = \frac{12 \mu L}{(1 - 0.63 (h/w))} \frac{1}{h^3 w}
\]

Equation 10

This equation is only valid if the height \( h \) is smaller than the width \( w \). If \( h \) is larger than \( w \), then \( h \) and \( w \) should be interchanged in Equation 10. For the resistance of the contraction parts, the same equation was used with the channel width \( w \) replaced by the average width of the contraction.

For channels in parallel, with resistances \( R_{\text{hyd},1} \) and \( R_{\text{hyd},2} \), the total resistance is

\[
\frac{1}{R_{\text{hyd,par}}} = \frac{1}{R_{\text{hyd},1}} + \frac{1}{R_{\text{hyd},2}}
\]

Equation 11

and for two channels in series, the total resistance is the sum of the resistances:

\[
R_{\text{hyd,ser}} = R_{\text{hyd},1} + R_{\text{hyd},1}
\]

Equation 12

Equation 11 and Equation 12 allow us to compute the resistance of any subset of channels within the one-channel device.

A schematic of the model, and the channel dimensions, are shown in Figure 17. Each of the channel sections is indicated by a resistance; the resistance of the bends connecting the channels was not accounted for explicitly, other than by adding to the length of the channel sections. This is a good approximation as long as the Reynolds number is small, i.e. \( \text{Re}<1 \)\(^{43}\), which is always true in our device.

The total flow rate through the device was assumed to be a constant equal to 1 \( \mu \text{L/min} \). The main channel trifurcates into two by-pass channel and a central channel. The ratio of
the resistances of the total central channel and one by-pass channel was computed to be 5.5. That means that the flow rate through the central channel is 1/12 of the total flow rate, i.e. 0.0838 μL/min. This corresponds to a mean velocity of 8.7 mm/s in the narrowest part. The pressure drop over the total device is 750 Pa, assuming a viscosity of \( \mu = 1 \text{ mPa·s} \). The Reynolds number, defined as \( \text{Re} = \frac{\rho V_m D_E}{\mu} \) with \( \rho = 1000 \text{ kg/m}^3 \) being the fluid density and \( D_E \) being the equivalent diameter\(^{10} \) has the value \( \text{Re} = 0.12 \) in the narrowest channels. Here, it is assumed that no cells are present in the device. Cells will increase the effective resistance in the channel. This will be accounted for in the analysis of the experimental results, in section 4.3.

![Figure 17: Schematic of the resistance analogue of the one-channel device. The table shows the channel dimensions. The colors in the schematic and the table correspond.](image)

**Finite element model**

To check our resistance analogue model and to obtain more detailed results, we carried out Finite Element Model (FEM) simulations using the commercial FEM package COMSOL. The design of the one-channel device was imported as an autocad file in COMSOL. Only half of the device was modeled, to reduce computational memory and time. The geometry was meshed in-plane (coordinates x-y) using triangular elements, and subsequently this mesh was extruded in the height (z-coordinate) direction to obtain 5 layers formed by prism elements, as can be seen in Figure 18. The mesh was refined in the narrow channel, as is shown in Figure 19. The total number of mesh elements was varied to ensure numerical convergence, up to the total number of 82840. The number of degrees of freedom was 1198920.

\(^{10}\) The equivalent diameter is the diameter of a circular duct or pipe that gives the same pressure loss as an equivalent rectangular duct or pipe.
The dimensions (width, height, length) of the channels were exactly those mentioned earlier in this chapter, see Figure 16. The no-slip condition was applied at the walls of the channels. At the inlet, a uniform velocity profile of 0.006 m/s corresponding to a flow rate of 1 µL/min was applied. At the outlet, a zero pressure condition was applied. The density and viscosity were set to the values of water, i.e. 1000 kg/m³ and 1 mPa·s. The COMSOL solver module used was the fluid dynamics module of the Multiphysics solver; the incompressible steady state Navier Stokes equation was solved.

Figure 19 shows results of the finite element calculations. The velocity profile shows the typical shape expected for a rectangular channel. Along a line in the z-direction (i.e. the height direction), the profile has a parabolic shape with a maximum velocity of about 5.5 mm/s in the by-pass channel, see Figure 20 (B), and about 18.2 mm/s in the narrow channel, see Figure 20 (D). From the profiles, the mean velocity was calculated by
integrating the simulated velocity over the entire cross section and dividing by the cross sectional area. This lead to a value of 8.72 mm/s in the narrow channels, which is in good agreement with the value of 8.7 mm/s, which we found from the resistance analogue plot as shown earlier in this section. The simulated pressure drop over the device equals 773 Pa, which is close to 750 Pa from the resistance analogue model. The agreement between the results of the resistance model and those of the FEM calculations is good. Hence, our conclusions based upon the resistance model are confirmed.

Figure 20: The velocity computed with the finite element model. (A): The magnitude of the velocity in cross sections of the channels; (B): The velocity profile in the by-pass channel along a line in the z-direction (i.e. the height direction), in the center of the channel; (C): The velocity profile in the narrow central channel along a line in the y-direction (i.e. the width direction), in the center of the central channel; (D): The velocity profile in the central narrow channel along a line in the z-direction (i.e. the height direction), in the center of the channel.
4.2 EXPERIMENTAL RESULTS

A variety of experiments with the one-channel device are carried out. The details of the experimental procedures and conditions are explained in Chapter 6. Here, only the essentials are explained.

Briefly, the channel walls were coated with Bovine Serum Album (BSA) by flushing a BSA solution through the device. BSA coats the surfaces of the channels and prevents any other protein adsorption, i.e. it prevents cell sticking. Cell solutions with 5 to $6 \times 10^5$ cells/mL THP-1 cells in a BSA-Phosphate buffered saline (PBS)-Ethylene diamine tetraacetic acid (EDTA) solution were introduced into the device at a total flow rate of 1 µL/min with the use of a syringe pump. PBS is a salty solution containing sodium chloride, sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate. The buffer helps to maintain a constant pH. EDTA is used as an agent which prevents cell sticking and which binds to calcium and magnesium ions and inhibits pseudopod formation and ensures that the cells maintain essentially spherical. The concentration of cells ensured that, on average, at each time 1 cell was present within the central narrow channel. Experiments were carried out with untreated THP-1 cells, and with THP-1 cells treated with 20 µg/mL Low Density Lipoprotein (LDL), with 100 µg/mL LDL, and with 4 nM PMA. These treatments mimic processes taking place during the development of atherosclerosis, as explained in Chapter 2. The procedure of the treatments is given in section 6.1. In two additional measurements, we coated the channel walls with VCAM-1 before carrying out the experiments. This is to study the effect of this adhesion molecule on untreated and on 100 µg/mL LDL-treated cells.

The complete duration of the experiments was approximately two hours. The cells were monitored using a high speed camera. We could not continuously record movies, due to restrictions of the camera. The obtained movies were analyzed to obtain cell size and velocities within the channel, using a specially developed image analysis code.

BSA-coated channels

Figure 21 to Figure 23 show snapshots of cells of varying sizes, flowing through the narrow channel (width 11.5 µm, height 14 µm), taken from the movies and compiled in one picture. The time interval between the snapshots depends on cell size, and is approximately 10 ms. The average size of the cells was between 12 and 14 µm. The snapshots show how the cells deform as they transit through the channel.

Figure 21 is the case for the untreated THP-1 cells flowing through the BSA-coated channel. For cell diameters similar to or smaller than the channel width, the cells hardly deform within the channel. For cell with diameters larger than 12 µm, however, the cell

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11 Pseudopod is a temporary projection of the cytoplasm of certain cells that serves in locomotion and phagocytosis.
starts to get a bullet shape, which is not directly relaxed after leaving the channel.\textsuperscript{12} This effect increases for larger cells. For cells smaller than 20 µm, the deformation does not show an evolution within the channel. Also, the velocity of the cells was found to be constant throughout the channel (see also Figure 63, in section 6.5). That means that no time-dependent behavior of the cells was observed in the experiments. For very large cells, i.e. larger than 20 µm, the area of contact between the cell and the channel walls becomes very large and the cells deform to a very large extent. Their shape is highly asymmetric, even leading to a concave trailing cell edge at the end of the channel. The extremely large cells were not included in the velocity analysis presented later on; their occurrence was very rare.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{untreated_THP1_cells}
\caption{Snapshots of the deformation of untreated THP-1 cells flowing through the narrow channel (width 11.5 µm, height 14 µm), for cell diameters vary from 10 µm to more than 20 µm.}
\end{figure}

The deformation behavior of the LDL-treated and the PMA-treated cells, shown in Figure 22 and Figure 23, do not show any qualitative difference with the non-treated cells. The morphology, however, does show some differences. The LDL-treated cells have more granules in the cytoplasm, visible as black spots in the pictures of Figure 22. This is due to the uptake of the LDL within the cell. The PMA-treated cells show a more irregular shape than the untreated ones, caused by microvilli\textsuperscript{13} on the membrane surface. This has been observed before, see e.g. Park et al.\textsuperscript{[26]}. These morphological changes, however, do not lead to significant changes in deformation behavior as mentioned before.

\textsuperscript{12} In the present study, we did not examine the recovery of the cells after exiting the narrow channels, although this could give important additional information about the rheological properties of the cell. In Chapter 7, Recommendations, we will come back to this point.

\textsuperscript{13} Microvilli are microscopic cellular membrane protrusions that increase the surface area of cells, and are involved in a wide variety of functions, including absorption, secretion, cellular adhesion, and mechanotransduction.
Figure 22: Snapshots of the deformation of THP-1 cells treated with 100 μg/mL LDL, flowing through the narrow channel (width 11.5 μm, height 14 μm), for cell diameters varying from 11 μm to 18 μm.

Figure 23: Snapshots of the deformation of THP-1 cells treated with 4 nM PMA, flowing through the narrow channel (width 11.5 μm, height 14 μm), for cell diameters varying from 11 μm to 17 μm.

Figure 24 depicts the measured cell velocity as a function of cell size for untreated THP-1 cells. The cell size was determined just before the cell entered the narrow channel, i.e. at a location at which the cell was not deformed (see Figure 21-Figure 23). Figure 24 shows the results of measurements carried out on two consecutive days in the same device. The velocity decreases with cell size, as expected. This dependency appears to be linear. The cell velocity ranges between 7.5 mm/s and 13 mm/s. In the previous section, we calculated the fluid velocity, without cells, to be 8.7 mm/s, which is in the range of measured cell velocities but at the lower end. The value of the measured cell velocities can be explained quantitatively using a model, which will be discussed in section 4.3.
The data shows a scatter of approximately 20%. The scatter may originate from various causes. First, since the channel height (14 µm) is larger than the average cell diameter for most cells, the precise location of the cells within the channel cross section may vary which could lead to a variation in velocity due to the parabolic velocity profile, see Figure 20. In addition, the image analysis resolution of 0.6 µm (one pixel) may lead to variations. Also, slight experimental variations such as in pressure, caused for example by the step motor of the syringe pump, could be a source of scatter. The measurements carried out at different days coincide within the scatter, which shows that the reproducibility of the experiment is good.

Figure 24: Cell velocity through the narrow channel (width 11.5 µm, height 14 µm) as a function of cell size for untreated THP-1 cells; measurements carried out on consecutive days. 83 cells were measured for day 1, 62 cells for day 2.

The measured velocity data for the treated cells are shown in Figure 25.

Figure 25: Cell velocity through the narrow channel (width 11.5 µm, height 14 µm) as a function of cell size for treated THP-1 cells. The number of cells measured were: 108 for 20 µg/mL LDL, 106 for 100 µg/mL LDL, and 102 for 4 nM PMA.
Again, the cell velocity appears to depend linearly on the cell size, with a smaller velocity for larger cells. The values are in the same range as for the untreated cells (here between 5 and 15 mm/s), and exhibit a similar scatter. There seem to be small differences between the various treatments, i.e. the 20 ug/mL LDL treatment appears to have slightly higher velocities than the other two treatment, however the difference is not significant considering the scatter of the data.

In Figure 26, the data of the treated and untreated cells are combined. All data are in the same range. Within the scatter, there is no difference between the different measurements. In other words, the treatments with LDL and PMA do not result in a significant change in cell velocity in the narrow channels.

Figure 26: Cell velocity through the narrow channel (width 11.5 µm, height 14 µm) as a function of cell size for untreated and treated THP-1 cells, for BSA coated channels. (Figure 24 and Figure 25 combined).

VCAM-1 coated channels

Snapshots of the deformation of the untreated THP-1 cells, as they flow through the VCAM-1 coated channel, are shown in Figure 27. The behavior appears to be not different from that of the untreated cells in the BSA-coated channel, see Figure 21. Hence, from the images there is no sign that the adhesion between the cells and the channel wall is influenced after the VCAM-1 treatment.
Figure 27: Snapshots of the deformation of untreated THP 1 cells, flowing through the VCAM-1 coated narrow channel (width 11.5 µm, height 14 µm), for cell diameters varying from 10 µm to over 20 µm.

The area of contact between the cells and the channel wall is estimated by manual inspection of the images obtained. The line of contact was measured from images such as in Figure 27, and the area was estimated by assuming a circular contact. The result for three cases, including BSA-coated and VCAM-1 coated channels and treatment with 100 ug/mL LDL, are shown in Figure 28. The contact area, obviously, increases with cell size. More importantly, the various cases do not differ from each other within the experimental scatter. This supports the speculation that the VCAM-1 treatment does not affect the cell adhesion to the wall. The scatter of the data and the fact that cells smaller than 11 µm still appear to have a finite contact area, show that the estimation has a rather poor accuracy.

Figure 28: The estimated contact area between the cells and the channel wall for untreated cells within a BSA coated channel, for untreated cells within a VCAM-1 coated channel and for cells treated with 100 ug/mL in a VCAM-1 coated channel.
Finally, Figure 29 shows the measured cell velocities as a function of cell size for untreated cells within a BSA coated channel, for untreated cells within a VCAM-1 coated channel and for cells treated with 100 ug/mL in a VCAM-1 coated channel. Again, no significant differences are observed.

Figure 29: Cell velocity through the narrow channel (width 11.5 µm, height 14 µm) as a function of cell size for untreated cells within a BSA coated channel, for untreated cells within a VCAM-1 coated channel and for cells treated with 100 ug/mL in a VCAM-1 coated channel.

The absence of any influence of the VCAM-1 treatment may be due to experimental factors. First, the BSA-PBS-EDTA buffer carrying the cells may remove or even coat over the VCAM coating on the walls. Second, the interaction between VCAM-1 and VLA-4, which is a ligand on the monocyte surface binding to the VCAM-1, may require ions to function. The latter may have been removed by the EDTA in the cell buffer since this binds calcium and magnesium ions, and therefore EDTA may function as an inhibitor for the VCAM-1 – VLA-4 interaction, see Table 1. To draw any firm conclusions, this needs further study.
Questions to be answered

The overall conclusion of the measurement is that none of the treatments result in a significant change in cell velocities in our device, even though we observe changes in morphology (for the LDL treatment and the PMA), as well as change in expressions of adhesion and activation markers, see Figure 5 and Figure 6. The results trigger the following questions:

- Is there really no change in mechanical cell properties due to the treatments, or is our device not sufficiently sensitive to detect the changes?
- If the latter is true, how can we modify our device and/or the experimental conditions to increase the sensitivity to mechanical changes?
- The cell size, however, has a significant effect on cell velocity. Can we explain this and, more particularly, can the linearity of the relationship be explained?

We will address these questions in the next section, in which we apply the model explained in Chapter 3 to our experimental results.
4.3 ANALYSIS OF THE EXPERIMENTAL RESULTS

The semi-empirical model of Lee & Fung\cite{33} based on dimensional analysis and experiments with artificial cells in cylindrical tubes, as discussed in detail in Chapter 3, was used to explain the experimental results. This means that the cell was effectively modeled as an elastic membrane with an effective elastic modulus, filled with an incompressible viscous liquid. The most important ingredients of the model used here are:

1. The relationship found between the cell velocity \( V_c \) and the mean flow velocity \( V_m \), shown in Figure 9. This figure is reproduced in Figure 30A. The relationship was found to be linear by Lee & Fung, and could be described with Equation 7, which reads:

\[
V_c = k(V_m - \alpha)
\]

The parameters \( k \) and \( \alpha \) were found to depend on the ratio of the cell size to the tube diameter \( \frac{D_c}{D_T} \), but almost independent of the dimensionless parameter \( \frac{\mu V_c}{E_c D_c} \), hence also of the cell’s elastic modulus \( E_c \) (see Lee & Fung\cite{33} and Sutera et al.\cite{34}, see Figure 12). The values of \( k \) and \( \alpha \) are listed in Table 2 (Chapter 2) for various cell to tube dimensions.

2. The presence of the cell in the tube was found to lead to an additional hydrodynamic resistance \( R_c \), compared to the resistance without the cell being present \( R_H \), which is given by, see Equation 8:

\[
\frac{R_c}{R_H} = \frac{D_T}{L_T} \frac{V_c}{V_m} f_3
\]

The (dimensionless) resistance function \( f_3 \) is plotted in Figure 10 and reproduced in Figure 30B.
The additional resistance depends on the ratio of the cell size to the tube diameter \( \frac{D_c}{D_T} \), as well as on the dimensionless parameter \( \frac{\mu V_c}{E_c D_c} \) (hence on cell stiffness).\(^{14}\) Effectively, the cell presence leads to an additional pressure drop that is given by, see Equation 8.

\[ \text{Figure 30: Main ingredients of the model of Lee & Fung}\(^{33}\) used in our analysis. A: cell velocity as a function of the mean flow velocity for various cell dimension / tube ratios; B: The non-dimensional resistance function } \( f_3 \).

To apply this model, we need to translate the dimensions of our channel, which has a rectangular cross section, to a tube diameter. For this, we use the equivalent diameter \( D_E \) for \( D_T \) in the entire analysis. We should note that this approximation becomes questionable for large aspect ratios of the channel. In our case, width \( w=11.5 \text{ µm} \), height \( h=14 \text{ µm} \), and \( D_E=13.1 \text{ µm} \).

\(^{14}\) This means that, compared to the full dimensional analysis leading to Equation 6, the dependency on the parameter \( \frac{L_T}{V_m} \left/ \frac{\mu_c}{E_c} \right. \) is neglected, which is justified if it is small since then the time scale of the convective flow is much smaller than the characteristic time scale of the cell, and time-dependent behavior of the cell does not occur. In our case, as mentioned in the experimental section 4.2, we indeed do not observe time-dependent effects except for very large cells. Also, a rough estimate of the dimensionless parameter shows that it is small in our case. Also, the direct adhesion between the cell and the channel wall, represented by \( f_{adh} \), is not taken into consideration. For the BSA coated channel, this is indeed a valid approximation.
Figure 31: Schematic of the analysis procedure used to calculate the expected cell velocity \( V_c \) from a combination of the resistance analogue model, the cell velocity model, see Figure 30A, and the cell resistance model, see Figure 30B. See the text for explanation.

Figure 31 shows the steps we used in the complete analysis procedure to calculate the expected cell velocity.

1. As a first step, we calculate the mean velocity in the central narrow channel, \( V_m(1) \), from the resistance analogue model of section 4.1, with as input the applied total flow rate (i.e. the inlet velocity), and the channel dimensions. The calculated velocity does not take into account the additional resistance of a cell yet. As has already been mentioned in section 4.1, for our one-channel device the result for the velocity is 8.7 mm/s, based on a total flow rate of 1 \( \mu \)L/min and the channel dimensions.

2. In step 2, the computed flow velocity \( V_m(1) \) is used as input for the model in Figure 30A and Equation 13 to calculate the cell velocity as a function of \( \frac{D_c}{D_T} \).

For this ratio, we took the values of 0.98, 1.13, 1.36, and 1.69, i.e. the values used by Lee & Fung. The result of this step in shown in Figure 32, where we have used the equivalent diameter of our channel \( (D_T=13.1 \mu m) \) to calculate the actual cell size. Note, that in this step we still assume that the cell does not affect the mean fluid velocity, other than by its dimensions.\(^ {15} \)

\(^ {15} \) This situation occurs, when a single channel without any bypass channels is controlled by a syringe flow rate and cells flow through this channel. The cell properties, other than its size, do therefore not change the cell velocity. Obviously, our situation is different; the presence of the bypass channels causes a redistribution of the flow due to the additional resistance of the cell, which depends on cell properties, and which causes the mean velocity in the central channel (and thus of the cell) to change. This effect is accounted for in steps 3 to 5 in our analysis.
Figure 32: Model result: cell velocity as a function of cell size, obtained after step 2 in our analysis. Note that the effect of the stiffness of the cell on the additional resistance is not taken into account in this figure.

3. Using the calculated cell velocity $V_c$ (1), the additional resistance caused by the cell presence is calculated in step 3, using the cell resistance model represented by Figure 30B and Equation 14. Here, the elastic modulus of the cell as well as that of the fluid viscosity plays a role, through the parameter $\frac{\mu V_c}{E_c D_c}$. For the elastic modulus, we used values in the range of 0.01 to 10 kPa. Typical values found for leukocytes are in this range, see Rosenbluth et al.\textsuperscript{[5]} and Appendix III. The result of this step is the dimensionless resistance function $f_3$ and the corresponding additional resistance $R_c$ due to the cell. The result of this step is shown in Figure 33. The relative additional resistance $R_c/R_H$ depends on the cell size and the dependency is stronger for higher elastic moduli. For small cell sizes (particularly close to the channel dimensions), the additional resistance is relatively low and not very strongly dependent on elastic modulus.

Figure 33: The computed relative additional resistance ($R_c/R_H$) due to the presence of the cell in our channel, as a function of cell size and cell elastic modulus.
4. Step 4 consists of a recalculation of the mean fluid velocity in the central narrow channel using the resistance analogue model, now taking into account the additional resistance induced by the cell obtained from the previous step and shown in Figure 33. Now, the effect of both the cell size as well as its elastic modulus is accounted for. The result is the fluid velocity $V_m(2)$ that depends on cell size and modulus.

5. The final step consists of recalculating the cell velocity using the calculated fluid velocity $V_m(2)$ and the model in Figure 30A and Equation 13. The result is the expected cell velocity $V_c$ as a function of cell size and elastic modulus (through the parameter $\frac{\mu V_c}{E_c D_c}$).

The final result is shown in Figure 34. The cell velocity appears to depend linearly on the cell size, the velocity decreasing for larger cells. Also, an increase in elastic modulus decreases the cell velocity. This effect is larger for larger cells. For cells having similar or smaller dimensions than the channel dimension (in this case the equivalent diameter is 13.1 $\mu$m), the dependency on elastic modulus becomes small. This general trend was also observed by Sutera et al.\textsuperscript{[34]} and Secomb & Hsu\textsuperscript{[41]}, see Figure 15.

![Figure 34: Final result of the model analysis: the expected cell velocity in our channel as a function of cell size and cell elastic modulus.](image)

In Figure 35, the model predictions are compared to the data measured for the untreated THP-1 cells (data from Figure 24). The agreement between the measurements and the prediction is remarkably good. Note, that we did not use any fit parameter to tune the model data, other than setting the elastic modulus to expected values. The measured data seem to be best described by a modulus between 0.1 and 1 kPa in the model. This fits the results of AFM measurements by Rosenbluth\textsuperscript{[5]} on leukocytes, see Figure 76 of Appendix III. Also, the comparison between the measurements and the data confirm the linear dependency of the cell velocity on the cell size. Considering the scatter of the experimental data, however, any modulus between 0.1 and 10 would fit the data reasonably well, particularly in the range of the measured cell sizes. An important conclusion of the model analysis is, therefore, that our measurements are not sufficiently sensitive to distinguish between elastic moduli of cells over a range of 2 orders of magnitude.
magnitude. Referring back to the measured data of the untreated and treated cells, this means that, even though the treatments could have lead to a change in elastic modulus, or more generally a change in mechanical properties, this would not show up in the measurements significantly, at least for our particular design and measurement conditions.

Figure 35: The model predictions for the cell velocity compared with the measured values for the untreated cells (data from Figure 24). We did not apply any fit parameter to the model data, other than setting the elastic modulus to expected values.

Now, we will apply the model to study the possibility of increasing the sensitivity to elastic modulus variations by changing the channel design and measurement conditions, in particular flow rate.

Figure 36 shows the cell velocity predicted by the model as a function of cell size, for varying channel size and two elastic moduli 0.1 and 1 kPa. The total inlet flow rate was kept constant at 1 µL/min. For all channel dimensions, the higher elastic modulus result in lower velocities, as expected. The magnitude of the change of the velocity due to the modulus change, however, depends on the channel dimensions. For smaller channel cross sections, the change is larger, in other words, thinner channels lead to a higher sensitivity for elastic modulus, for a certain cell size. This can be clearly seen in Figure 37, which plots the relative change in cell velocity \(^{16}\) when the elastic modulus changes from 0.1 to 1 kPa (i.e. the relative difference between the lighter lines with “filled” squares and the darker lines with “not filled” squares in Figure 36, indicated by the arrows). A change in the smaller of the two dimensions (height or width), has a stronger effect than a change in the larger dimension. The reason is that the channel’s hydraulic resistance is more sensitive to a change in the smaller dimension (see Equation 10), which affects the mean fluid velocity and hence the cell velocity. An additional effect that contributes is that the

\(^{16}\) For example, the cell with a size of 15 µm and with an effective elastic modulus of 0.1 kPa has a velocity of A and with an effective elastic modulus of 1 kPa a velocity of B. Relative change in cell velocity when the elastic modulus changes from 0.1 to 1 kPa is calculated as follows: absolute value of \((A-B)/A\).
cell velocity dependence on cell size is also stronger for the smaller channels, at equal flow rate, i.e. the slopes of the lines in Figure 36 are steeper for the smaller channels. The conclusion is that channels with a smaller cross section give a higher sensitivity to the cell’s elastic modulus. A disadvantage of having smaller channels is the much higher cell velocity, which makes a practical image analysis less accurate. Also, smaller channels have an increased chance of cell blockage.

![Figure 36: Predicted cell velocity as a function of cell size for various channel dimensions and elastic moduli; the total flow rate is constant at 1 µL/min.](image1)

![Figure 37: Predicted change in cell velocity when the elastic modulus changes from 0.1 to 1 kPa, for various channel dimensions (extracted from Figure 36).](image2)

The influence of the flow rate is shown in Figure 38. In this case, the channel cross section is kept constant at \( w = h = 8 \) µm. Obviously, a lower flow rate leads to a lower cell velocity. The dependence of cell velocity on cell size is stronger for the high flow rate.
than for the low flow rate. The influence of a change in elastic modulus (from 0.1 to 1 kPa), is, in absolute sense, smaller for the low flow rate condition, however the relative change is substantially larger. This can be seen in Figure 39 which plots the relative change in cell velocity when the elastic modulus changes from 0.1 to 1 kPa. The green and the purple line correspond to the data in Figure 38. For a cell with a diameter of 14 µm, the relative change in cell velocity is 35% for the flow rate of 0.1 µL/min, but for 1 µL/min less than 20%. The conclusion is that lower flow rates lead to a higher sensitivity to elastic modulus of the cell. A disadvantage of lower flow rate is that it is more difficult to introduce the cells into the device. In our device, for example, flow rates lower than 1 µL/min lead to sedimentation and sticking of cells resulting in almost zero cell entry into the device.

Figure 38: Predicted cell velocity as a function of cell size for various flow rates and elastic moduli; the channel dimensions are kept constant at $w=h=8\,\mu m$.

Figure 39, finally, also shows the effect of various channel configurations, such as two narrow channels or three narrow channels instead of a single one. The three-channel configuration is illustrated in Figure 40. This configuration is a step from our one-channel device to our capillary network device discussed in Chapter 5. Figure 39 proves that the sensitivity for elastic modulus changes of the cell velocity increases with the number of parallel channels. The effect of an increase of the number of parallel channels is, in fact, equivalent to decreasing the flow rate within the individual channels, at an equal global flow rate, shown in Figure 38. Ultimately, the capillary network device should therefore be more sensitive to elastic modulus changes. The results of the measurements will be shown in Chapter 5.

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17 This explains the difference in slopes observed in Figure 53 of Chapter 5, for the capillary network device.
Figure 39: Predicted change in cell velocity when the elastic modulus changes from 0.1 to 1 kPa for various channel configurations. For the 1-channel case the flow rate is varied from 0.1 to 1 µL/min. The channel dimensions are kept constant at $w=h=8 \, \mu m$.

Figure 40: Resistance analogue sketch illustrating the three parallel channel configuration.

In the end, we can now answer the questions posed at the end of the previous section:

- Our measurements are not sufficiently sensitive to distinguish between elastic moduli of cells over a range of 2 orders of magnitude. This means that, even though the treatments could have lead to a change in elastic modulus, or more generally a change in mechanical properties, this would not show up in the measurements significantly, at least for our particular design and measurement conditions.
- The device and the experimental conditions can be optimized to increase the sensitivity to mechanical changes: the channel cross section should be decreased, the flow rate should be decreased, or the number of parallel channels should be increased. It should be noted, though, that all these measures will be bounded by practical limits: manufacturability, image analysis resolution, cell inflow limitations, syringe pump resolution, and possible cell blockage effects.
- The measured cell size effect can be quantitatively described by the model. The linearity of the cell velocity – cell size relationship is caused by the interplay between the additional resistance introduced by the cell presence and the redistribution of flow between the central channel and the by-pass channels.
Discussion

The agreement between the experimental results and the model predictions of Lee & Fung\textsuperscript{[33]}, shown in Figure 35, is remarkably good, even though the model contains some course approximations to our real conditions. The most important approximations are the following:

- The undeformed shape of the model cells of Lee & Fung\textsuperscript{[33]} and Sutera et al.\textsuperscript{[34]} is bi-concave, mimicking red blood cells, rather than our monocytes that are spherical. Notwithstanding, the deformation of the model cells in the tubes is remarkably similar to the deformation of the monocytes in our channel, see Figure 11 and Figure 21.
- The channel of Lee & Fung\textsuperscript{[33]} is cylindrical rather than rectangular as in our case. We, in a course way, accounted for this by using the equivalent diameter of our channel. This can be expected to be a reasonable approximation for relatively small aspect ratios.
- The model cells are rubber membranes with an effective elastic modulus, filled with an incompressible viscous liquid. The cell viscosity, however, is not explicitly accounted for in the model. As mentioned before, in our experiments the cell viscosity is not expected to play an important role (see footnote 14). The membrane thickness of the model cells of Lee & Fung is rather large, i.e. 0.01 of the cell size. Membranes of real cells are significantly thinner, typically a factor of 4 (see Seshadri et al.\textsuperscript{[38]}). In our case, we may have compensated both for the effect of the viscosity as well as for the membrane thickness, by tuning the value of the elastic modulus. We do not expect, however, that the trends we find will be substantially affected by these approximations.
- The model does not include direct interactions between the cell and the channel wall. Implicitly, Lee & Fung assumed that the sphere was separated from the wall by a thin liquid lubrication layer all the time. This is most probably a good assumption for our experiments with the BSA coated device. For the VCAM-1 coated device, however, we would expect the direct interaction between the cell and the channel wall to become important. The fact that we do not see a big influence of the VCAM-1 treatment, and the model describes the data well, suggest that either the interaction does not influence the velocity, or the interaction is really absent. The latter would be the case if, in reality, the VCAM-1 coating process was not successfully done.

The ability of the model to capture the basic features of our experiment is supported by the result shown in Figure 41. This concerns an experiment carried out in another device than for all other results described up to now in this chapter, in a channel with a different cross section, namely with a width \( w = 10 \, \mu m \) and a height of \( h = 12 \, \mu m \). An identical model prediction has been carried out for this case, and, as Figure 41 shows, the experimental data are well predicted also in this case. For completeness, Figure 42 shows the predicted cell velocities for the two experimental devices we have used, for two elastic moduli. There is a clear influence of the dimensions.
Figure 41: Model predictions for the cell velocity compared with the measured values for the untreated cells for a channel with a cross section of $w=10\, \mu m$ and a height of $h=12\, \mu m$. The inlet flow rate was $1\, \mu L/min$.

Figure 42: Predicted cell velocity as a function of cell size for the two channel dimensions used in the experiments, for two elastic moduli; the total flow rate is constant at $1\, \mu L/min$. 
4.4 CONCLUSIONS

The velocities of the cells in the micro-fluidic one-channel device were successfully determined. We did not encounter any problems caused by clogging of cells, which is probably due to the gradual contraction. None of the treatments mimicking processes in atherosclerosis, though, resulted in a significant change in cell velocities in our device, even though we observe changes in morphology (for the LDL and the PMA treatment), as well as changes in expressions of selected adhesion molecules and activation markers, see Chapter 2.

We used the semi-empirical model of Lee & Fung to predict the measured cell velocities. Even though the model contains course approximations, the agreement between the model prediction and the measured data was good. An important conclusion of the model analysis is, however, that our measurements are not sufficiently sensitive to distinguish between effective elastic moduli of cells over a range of 2 orders of magnitude. This means that, even though the treatments could have lead to a change in elastic modulus, or more generally a change in mechanical properties, this would not show up significantly in the measurements, at least for our particular design and measurement conditions.

The device and the experimental conditions can be optimized to increase the sensitivity to mechanical changes: the channel cross section should be decreased, the flow rate should be decreased, or the number of parallel channels should be increased. It should be noted, though, that all these measures will be bounded by practical limits: manufacturability, image analysis resolution, cell inflow limitations, syringe pump resolution, and possible cell blockage effects.

We carried out measurements with another device which has a capillary network consisting of 32 narrow parallel channel and two large by-pass channels, to investigate the sensitivity of a parallel channel configuration to a change of mechanical cell properties. This is the topic of the next chapter.
5. CAPILLARY NETWORK DEVICE

In this chapter, the capillary network device is described, and an analysis of the expected flow within the device with the use of an analytical and a numerical model are studied. Then, the experimental results are presented and analyzed.

The design

Our second design, shown in Figure 43, consists of a network of continuously splitting and narrowing microchannels that mimic the capillary network in vivo. Compared to the one-channel device discussed in the previous chapter, the single channel is thus replaced by a network of capillaries that consist of 32 narrow parallel channels. As argued in Chapter 4, multiple parallel channels will increase the sensitivity of the device to mechanical changes of the cell, for the same global flow rate. Another important advantage of the device is that it enables the simultaneous measurement of multiple cells in the various channels. From an application point of view, this makes high throughput analysis possible, which is relevant for clinical applications. The network is surrounded by larger by-pass channels. The flow thus trifurcates which enables to reach lower flow velocities in the capillary network at higher applied flow rates through the inlet. Also, the by-pass channels prevent a large pressure drop over the device, which in practice helps to avoid delamination of the device. The narrowest channels have a width of 8 µm and the large by-pass channels have a width of 200 µm. The height of all channels is 17.5 µm. The design was inspired by the work of Rosenbluth et al. The main difference with Rosenbluth is that their design has 64 narrow branches and their channels are narrower (5.8 µm wide and 13.3 µm high).

Figure 43: Design of the capillary network device.
5.1 FLOW ANALYSIS OF THE DESIGN

Just as for the one-channel device, we have used a resistance analogue model to analyze the flow behavior of the one-channel design. The model was explained in section 4.1. The hydrodynamic resistance of each channel section is given by Equation 10.

A schematic of the model, and the channel dimensions, are shown in Figure 44. Each of the channel sections is indicated by a resistance; the resistance of the bends connecting the channels was not accounted for explicitly, other than by adding to the length of the channel sections. This is a good approximation as long as the Reynolds number is small, i.e. \( \text{Re}<1 \) \[^{43}\], which is always true in our device.

![Figure 44: Schematic of the resistance analogue of the capillary network device. The table shows the channel dimensions. The colors in the schematic and the table correspond.](image)

The total flow rate through the network was assumed to be a constant equal to 5 \( \mu \text{L/min} \). The main channel trifurcates into two by-pass channels and a capillary network. The ratio of the resistances of the capillary network and one by-pass channel was computed to be 3. That means that the flow rate through the capillary network is 1/7 of the total flow rate, i.e. 0.7 \( \mu \text{L/min} \). Consequently, the flow rate through a single narrow channel in the middle of the network is 1/32 of 0.7 \( \mu \text{L/min} \), i.e. 0.022 \( \mu \text{L/min} \). This corresponds to a mean velocity of 2.7 mm/s. The pressure drop over the total device is 647 Pa, assuming a viscosity of \( \mu=1 \) mPa·s. The Reynolds number, defined as \( \text{Re} = \rho V_m D_E / \mu \) with \( \rho=1000 \) kg/m\(^3\) being the fluid density and \( D_E \) being the equivalent diameter, has the value \( \text{Re}=0.034 \) in the narrowest channels. Here, we have assumed that no cells are present in the device.

When a cell solution is flushed through the device, cells may in practice clog part of the capillary network as shown in Figure 45, which changes the characteristics of the device. The resistance analogue model enables us to study the effect of permanent cell clogging.
by setting the resistance of the clogged channel section to infinity, and recalculating all
the resistances and corresponding velocities.

Figure 45: Picture of cells clogging the capillary network. Four channels are clogged; one cell is
passing through another channel.

Four situations are shown in Figure 46, in which different branches of the network are
clogged. Table 3 shows the computed velocities in the narrowest channels in the branches
indicated in Figure 46 for the various clogging situations. Also, the ratio of the
hydrodynamic resistance in the capillary network to that in the by-pass channel is
indicated, as well as the pressure drop over the capillary network. The numbers 1, 2, 3 in
the figure and in the table refer to different sections in the network.
Figure 46: The resistance network model, with different branches of the network clogged.

The velocity in the narrow channels increases by the clogging by a value that depends on the specific location of the clogging. Even in the case of only 4 out of 32 narrow channels being clogged, the local velocity can be increased by as much as 30% (as is the case for branch 2 in case A). The largest increase in velocity for the cases considered in Figure 46 is 48% (case D, in which 8 narrow channels are clogged). Obviously, the increase will be even larger when more channels are being clogged.

The change in pressure drop not excessive, as it can be seen in Table 3 (up to 2% for case C). Also, the change in the ratio $R_{\text{hyd, cap}}/R_{\text{hyd, hyp}}$ is smaller than the velocity change (up to 17% for case C). The result is that the total flow through the capillary network will not be changed much. The biggest effect of the clogging is a redistribution of the liquid over less channels than for the unclogged case, leading to a locally higher velocity. Irrespective if the flow is pressure or flow rate controlled, the effect of the clogging is the same.

The local velocity increase is not only dependent on the number of channels being clogged, but also on the location of the clogging. This can be seen from a comparison between B, C, and D, for all of which the number of clogged channels is 8, however the
effects on velocity are completely different, as listed in Table 3. Also, the velocity increase is not uniform over the complete device. Generally, due to the interconnection between the channels, the effect is larger for channels closer to the clogged channels.

Table 3: The influence of clogging on the velocities in the narrowest channels of various sections of the capillary network, on the ratio of hydrodynamic resistance in the network over that of the bypass channel, and on the pressure over the capillary network. The situations are sketched in Figure 46.

<table>
<thead>
<tr>
<th></th>
<th>V [mm/s] (1)</th>
<th>V [mm/s] (2)</th>
<th>V [mm/s] (3)</th>
<th>$R_{\text{hyd,cap}}/R_{\text{hyd,by}}$</th>
<th>$p_{\text{cap}}$ [Pa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>unclogged</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>3</td>
<td>647</td>
</tr>
<tr>
<td>(A)</td>
<td>2.7</td>
<td>3.5</td>
<td>3.0</td>
<td>3.1</td>
<td>652</td>
</tr>
<tr>
<td>(B)</td>
<td>3.6</td>
<td>3.1</td>
<td>-</td>
<td>3.3</td>
<td>657</td>
</tr>
<tr>
<td>(C)</td>
<td>2.9</td>
<td>3.6</td>
<td>-</td>
<td>3.5</td>
<td>661</td>
</tr>
<tr>
<td>(D)</td>
<td>2.8</td>
<td>4.0</td>
<td>4.0</td>
<td>3.4</td>
<td>658</td>
</tr>
</tbody>
</table>

This property of the capillary network device has important consequences for its practical use. Clogging, whether it is temporary or permanent, will always occur in practice. Therefore, the fluid velocities in the narrow channels will change substantially during a measurement, possibly in a fluctuating way. The transit times of the cells, in which we are interested, will obviously depend not only on their mechanical properties but also on the velocity of the carrier fluid, and it will therefore also change substantially during a measurement. This effect is unpredictable and, for a proper interpretation of the measurement, should be continuously corrected for. The latter is practically very cumbersome, and a device design is preferred that does not have this disadvantage. This difficulty was not observed in the one-channel design presented in Chapter 4.

**Finite element model**

To check our resistance analogue model and to obtain more detailed results, we carried out Finite Element Model (FEM) simulations using the commercial FEM package COMSOL. The design of the capillary network was imported as an autocad file in COMSOL. Only half of the device was modeled, to reduce the required computational memory and time. The geometry was meshed in-plane (coordinates x-y) using triangular elements, and subsequently this mesh was extruded in the height (z-coordinate) direction to obtain 5 layers formed by prism elements, as can be seen in Figure 47. The mesh was refined in the narrowest channels, as is shown in Figure 48. The total number of mesh elements was varied to ensure numerical convergence, up to the total number of 72,625. The number of degrees of freedom was 1,079,220.
Figure 47: Computational domain (top) and finite element mesh (bottom) used in the COMSOL finite element simulations. Only half of the device is shown.

The dimensions (width, height, length) of the channels were exactly those shown in Figure 44 for the resistance network model. The no-slip condition was applied at the walls of the channels. At the inlet, a uniform velocity profile of 0.0238 m/s corresponding to a flow rate of 5 µL/min was applied. At the outlet, a zero pressure condition was applied. The density and viscosity were set to the values of water, i.e. 1000 kg/m³ and 1 mPa·s. The COMSOL solver module used was the fluid dynamics module of the multiphysics solver; the incompressible steady state Navier Stokes equation was solved.

Figure 48: Detail of the finite element mesh used in COMSOL.
Figure 49 shows results of the finite element calculations.

Figure 49: The velocity computed with the finite element model. (A): The magnitude of the velocity in cross sections of the channels; (B): The velocity profile in the by-pass channel along a line in the z-direction (i.e. the height direction), in the center of the channel; (C): The velocity profile in the narrow channels along a line in the y-direction (i.e. the width direction), in the center of the channel. (D): The velocity profile in the narrow channels along a line in the z-direction (i.e. height direction), in the center of the channel.

The velocity profile shows the typical shape expected for a rectangular channel. Along a line in the z-direction (i.e. the height direction), the profile has a parabolic shape with a maximum velocity of about 14 mm/s in the by-pass channel, see Figure 49 (B), and about 5 mm/s in the narrow channels, see Figure 49 (D). Since the height of the narrow channels (17.5 µm) are relatively larger than their widths (8 µm), the profile along a line in the z-direction, is blunter than the profile along a line in the y-direction (i.e. the width direction), see Figure 49 (C) and Figure 49 (D). From the profiles, the mean velocity was calculated by integrating the simulated velocity over the entire cross section and dividing by the cross sectional area. This lead to a value of 2.62 mm/s in the narrow channels, which is in good agreement with the value of 2.7 mm/s, which we found from the resistance analogue plot as shown earlier in this section. The simulated pressure drop over the capillary network equals 677 Pa, which is close to 647 Pa from the resistance analogue model. This is close to the value of the pressure drop over the one-channel design.
The FEM model was also used to estimate the effect of cells clogging the channels. In particular, the situation (B) in Figure 46 was modeled. The results are shown in Table 4. The agreement between the results of the resistance model and those of the FEM calculations is good. Hence, our conclusions based upon the resistance model are confirmed.

Table 4: Average velocity in the narrow channels for the resistance model (RM) and for the finite element model (FEM), both for the unclogged case and for the clogged case of Figure 46 (B).

<table>
<thead>
<tr>
<th></th>
<th>V [mm/s] (1)</th>
<th>V [mm/s] (2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unclogged, RM</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td>Unclogged, FEM</td>
<td>2.62</td>
<td>2.62</td>
</tr>
<tr>
<td>Clogged (B), RM</td>
<td>3.6</td>
<td>3.1</td>
</tr>
<tr>
<td>Clogged (B), FEM</td>
<td>3.58</td>
<td>3.03</td>
</tr>
</tbody>
</table>
5.2 EXPERIMENTAL RESULTS

We carried out experiments with the capillary network device by flushing through a BSA-PBS-EDTA solution containing 5 to 6·10^5 cells/mL untreated THP-1 cells. The details of the experimental procedures and conditions are explained in Chapter 6. Briefly, after coating the channel walls with BSA, a constant syringe pump flow rate of 5 µL/min was used to introduce the cell solution into the device. The complete duration of the experiments was approximately two hours. The cells were monitored using a high speed camera. The obtained movies were analyzed to obtain cell size and velocities within the channels, using a specially developed image analysis code.

Figure 50 shows the result of one experiment and plots the cell velocity as a function of cell size. The cell size was determined when the cell was passing through the 20 µm wide channel sections, indicated in Figure 51. It was observed that the cells are circular in 20 µm section, as they were not deformed. The two lines in Figure 50 correspond to the channel sections with widths of 12 µm and 8 µm, respectively, see Figure 51. 144 cells were measured during the experiments.

Some general observations can be done from Figure 50. First, as expected, the cell velocity in the narrower channels is smaller than in the wider channels. The reason for this is that the wider channels split into two to form two narrower channels. Second, larger cells have a lower velocity. The dependency on cell size is stronger for the 12 µm channels than for the 8 µm channels; this can also be expected and the explanation is given in section 4.3. The cell velocity in the 8 µm channel varies between about 0.5 mm/s and 3 mm/s. From the capillary network model explained in the previous section, we expected a fluid velocity, without cells, of 2.7 mm/s, which is within the range of the measured cell velocities but at the high end. This is also understandable since the presence of a cell will increase the effective flow resistance and therefore reduce the mean fluid velocity. The models of cells flowing through capillaries, explained in Chapter 3, show that the cell velocity will be larger than the mean fluid velocity, particularly for small cells. The data of Figure 50 are also consistent with this expectation as well.
A proper quantitative analysis of the data of Figure 50, however, is hindered by the large scatter of the data, which is as high as 100% or more, which is substantially higher than the scatter in the one-channel device, which was found approximately 20%. The reason for the scatter is the temporary and permanent blockage of the channels by cells clogging the entrances of the 8 μm channels. This effect can be seen in Figure 51. The two images A and B were taken at different times during the experiment and they illustrate the change of blockage in time. As we have seen from the flow calculations of the capillary network device, presented in the previous section, blockage of part of the device can indeed substantially change the distribution of the fluid flow rate in the channels. In practice, this effect dominates the behavior of the cells to such an extent, that the analysis of the influence of mechanical properties of cells on the velocities becomes very inaccurate for the capillary network design.

Figure 51: Images of the capillary network device, showing cells blocking the entrances of some of the narrowest channels. In A, the widths of the various channel sections are indicated. A and B were taken at different times during the experiments. The blockage changes in time.

Still, the level of blockage in the experiment of Figure 50 and Figure 51 is relatively mild. The amount of blockage was highly variable. In Figure 52 a more severe blockage as can be seen. In this image, only three channel sections are not blocked: B indicates a pair of neighboring unblocked channels, whereas A indicates an isolated unblocked narrow channel.
Figure 52: Image of the capillary network device, showing cells blocking the entrances of many of the narrowest channels. A and B indicate unblocked channels for which the cell velocities were measured, see Figure 53.

The cell velocities in these channel sections, as a function of cell size, are plotted in Figure 53.

![Graph showing cell velocity as a function of undeformed cell size](image)

Figure 53: Cell velocity as a function of undeformed cell size, for THP-1 cells in the capillary network device. The two lines correspond to different 8 µm channel sections in the network, see Figure 52.

Clearly, the difference in cell velocity in the channel sections is very large especially for the smaller cells, and this effect is solely caused by the blockage. This confirms experimentally the analysis of the effect of blockage on the mean fluid velocity, carried out by the flow analysis model explained in the previous section. A detailed quantitative comparison between the flow analysis model and the experiment cannot be given, however, since (i) in the experiment we only observe part of the capillary network (i.e. 20 channels of the 32), and (ii) we measure the cell velocity, not the fluid velocity that is
obtained from the model. Considering the inaccuracy of the measurement data, we did not analyze the results any further with use of the model of Lee & Fung\textsuperscript{[33]}, which we did for the one-channel device, see Chapter 4.

5.3 CONCLUSIONS

The main conclusion from both the computations and the experiments is that our capillary network device is not suitable for the reliable study of the mechanical properties of cells by observing the cell velocities. The variations in cell velocity caused by temporary or permanent blockage of parts of the network, which is unavoidable in practice, are so large that possible effects of mechanical properties may be hidden in the scatter. The one-channel design discussed in Chapter 4 did not show any cell blockage problems, which is probably due to the more gradual contractions between different channel widths. However, even with more gradual contractions, the capillary network will always have the issue that a cell passing through one channel creates a temporary blockage that affects the cell velocity in other parallel channels, resulting in scatter. This is a major disadvantage that works against the advantage of the expected larger sensitivity to changes in mechanical properties of cells, compared to the one-channel device.
6. EXPERIMENTAL DETAILS

In this chapter, all the experimental details of our work are described, including the cells and their treatments, the manufacturing of the devices, the experimental set-up and procedures, and the image analysis. This chapter may be consulted by a reader who wishes to know more details; the basics have been already been included in Chapters 4 and 5, so that these can be understood without referring to the present chapter.

6.1 THE PROCEDURE OF CELL CULTURE AND TREATMENTS

Cells from a human acute monocytic leukemia cell line\(^1\), THP-1 cells, were used in this study. It should be noted that while THP-1 cells are called monocytes, they are actually more representative of monoblasts, a slightly immature form of monocytes (see Appendix I for a classification of blood cells). THP-1 monocytes were obtained and cultured at sub-confluence\(^1\) according to the supplier’s recommended specifications (ATCC). Briefly, cells were seeded at a concentration of \(2 \times 10^5\) cells/mL in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS), glutamax (2 mM/mL, Invitrogen) and antibiotics (streptomycin and penicillin, 100 units/mL, Invitrogen). Cells were sub-cultured every 2-3 days, ensuring that the cell concentration did not exceed \(1 \times 10^6\) cells/mL (over confluence), by pelleting the cells via centrifugation at 1000 rpm for 5 min., discarding the old medium, and finally diluting the cells in fresh medium to \(2 \times 10^5\) cells/mL. THP-1 cells were treated (co-cultured) in the presence of 20 or 100 \(\mu\)g/mL Low Density Lipoprotein (LDL) (invitrogen), and 4 nM phorbol 12-myristate 13-acetate (PMA) (Sigma) for 24 hours prior to experimental study. 4 nM PMA allows for maturation of THP-1 cells towards macrophages, without a full differentiation. Mechanically scraping of cells which is widely used to get the differentiated THP-1 cells off the substrate, but it was prevented here by using only the suspended THP-1 cells. For the measurement of expression changes of adhesion molecules and activation markers on THP-1 cells, commercially available antibodies were used, which were fluorescence labeled with a fluorophore. They were mixed with the cells for about 10 min. Before experimental study, any excess LDL, PMA or antibody in the medium were washed away with Phosphate buffered saline (PBS) by two centrifugation steps. PBS is a salty solution containing sodium chloride, sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate. The buffer helps to maintain a constant pH.

The cells from the culture were used in our micro-fluidic devices according to the procedure described in section 6.4.

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\(^1\) Cells isolated directly from a person (known as primary cells) have a limited lifespan. After a certain number of cell divisions the cells stop dividing, while retaining viability. Therefore, in our experiments a human acute monocytic leukemia cell line, THP-1, was used that was isolated from a leukemia patient. This cell line divides indefinitely while their properties are maintained.

\(^1\) Sub-confluence means that the cells don't form a close layer of cells yet (confluent). They are believed to grow at their fastest speed (log phase). If the cells are confluent, some cellular processes are slowed down or even shut down.
6.2 MANUFACTURING OF MICROFLUIDIC DEVICES

Our micro-fluidic channels were made by positioning polydimethylsiloxane (PDMS) structures on a glass substrate. Figure 54 shows the basic steps of the manufacture processes. PDMS is a very well known material for micro-fluidic devices with advantages of flexibility, transparency and ease of replication with the use of a master with the inverse (or photographic "negative") of the pattern (channels) to be transferred. As a master for replication we used SU-8, see below. Another advantage of PDMS is that it is permeable to air, which turned out to be convenient in removing air bubbles from the channels during the experiments.

Figure 54: The basic steps of the manufacturing processes of our micro-fluidic devices.

The manufacturing procedure of our devices is explained below in a detailed way. It consists of PDMS sample preparation and assembling the parts.

**PDMS sample preparation**

First a glass plate which is used as a substrate for applying for the negative photoresist, SU-8, is cleaned with soap and water to remove particles on the surface as well as any traces of organic, ionic, and metallic impurities. Then the substrate is rinsed with Iso-Propyl Alcohol and dried to remove the absorbed water on the surface to promote the adhesion of SU-8, since hydrated substrates result in adhesion failures. Subsequently, it is treated with O₂ plasma (600 W) to remove residual organic contamination. Then, a standard SU-8 process is performed. SU-8 is a negative photoresist. It is a very viscous polymer that can be spincoated on a substrate with a thickness ranging from 1 micrometer up to 2 millimeters, and still can be processed with standard lithography. Its maximum absorption is for ultraviolet light with a wavelength of 365 nm. When exposed, SU-8's long molecular chains cross-link causing the solidification of the material.
The procedure we used to make the SU-8 master is as follows:

- **Spin coating:** SU-8 is spincoated on the glass plate, see Figure 54 (a), firstly at 500 rpm for 10 seconds and then at 1200 rpm for 45 seconds, to obtain the required layer thickness.

- **Soft bake:** The glass substrate with SU-8 on top is soft baked for 5 minutes at 65 °C and 15 min at 95 °C. This step is done to drive off excess solvent and to promote the adhesion of the resist to the glass substrate.

- **Exposure:** The SU-8 layer is exposed through a mask four times for 9 seconds at 10 mJ/cm² of UV light with a wavelength 365 nm to transfer the desired pattern from the mask, see Figure 54 (b). The mask is a glass substrate with chromium patterns of the desired geometries.

- **Post exposure bake:** This is performed for 1 minute at 65 °C and for 5 minutes at 95 °C. It removes residual solvents and anneals the film to promote the adhesion of the resist.

- **Development:** The unexposed SU-8 areas are removed with a developer (mrDEV600, Microresist US), since SU-8 is a negative photoresist in photolithography, i.e. the exposed parts will crosslink and solidify.

- The glass plate with SU-8 structures is rinsed with Iso-Propyl Alcohol, washed with water, and dried at 180 °C for 1 hour, see Figure 54 (c).

Our micro-fluidic devices can be replicated by casting silicone elastomer, i.e. PDMS resin over the SU-8 on the glass substrate. This procedure is explained below:

- **The used silicone elastomer is the Slygard 184 A+B from Dow Corning.** The compounds A+B are thoroughly mixed by stirring at the ratio of 1:9, following the description of the company.

- **The mixture is evacuated for 5 minutes to avoid air pockets in the cured silicone, since enclosed air pockets would lead to a porous matrix and defects in the replicas.**

- The mixture, i.e. liquid PDMS is poured on the SU-8 and then it is evacuated for 5 minutes again, see Figure 54 (d).

- **PDMS is cured at 65 °C for 4 hours and can then be easily peeled off from the SU-8 master.**

- **PDMS is kept on the SU-8 master until coupling with a glass substrate with inlet and outlet holes (see assembly). This is the best way to keep the active/fluidic side clean.**
Assembly

- The inlet and the outlet holes are sandblasted in a glass substrate using a small sample holder, and a nozzle with compressed air and sand (Al₂O₃ grains with an average diameter of 30 µm).
- The glass substrate with inlet and outlet holes is treated in a UV-ozone photoreactor.
- The glass substrate is placed on the top of the Fineplacer (96"Lambda"), that is used for alignment of substrates.
- The PDMS is peeled from the SU-8 master and the PDMS-side to be adhered to the glass is treated with corona. During “corona” treating, the material being treated is exposed to an electrical discharge, or "corona." Oxygen molecules within the discharge area break into their atomic form and are free to bond to the ends of the molecules in the material being treated, resulting in a chemically activated surface. A hand held corona device (BD20-AC) is used, see Figure 54 (e).
- The PDMS sample is placed on the bottom chuck of the fine placer.
- The PDMS and glass substrate are aligned and coupled.
- The sample is placed in an oven for about three hours at 100 °C to increase the bonding strength further.
- The Glass/ PDMS edge is glued with UV-curing Dymax M-T.
- A groove is sandblasted on another glass plate for glass fibers and it is cleaned in UV-ozone.
- The glass plate with the grooves is positioned on the backside of the sample over the sandblasted holes and glued with Dymax 3016 to the sample.
- The glass fibers (SGE Fused Silica Tubing (100% Methyl Deactivated)) with polyimide coating and with an inner/outer diameter of 200 µm/363 µm) are glued in the groove with Dymax M-T and the glue is crosslinked with UV-light.
- The assembly is finished by gluing the fiber/glass side with another glue called UHU to prevent airbubbles leakage along the fibers, see Figure 54 (e) and Figure 55.

Figure 55: Our micro-fluidic device.
Measurement of the height of the channels

The height of the channels, the width of the narrowest channel and the width of the bypass channels of one-channel device were designed to be 15 µm, 10 µm, and 150 µm respectively. Due to details in the manufacturing process, however, the actual height deviated from the designed height. The real dimensions of the channels were measured using confocal microscopy. The procedure is as follows: Fluorescent solution of AlexaFluor 488 (Invitrogen) is flushed through the device for 10 minutes with 1 µL/min. By using a confocal microscope the channel with the fluorescent solution is illuminated by a laser beam with a wavelength of 488 nm and the emitted fluorescent light from the channels is detected by a photo-detection device transforming the light signal into an electrical one that is recorded by a computer. The actual width of the narrowest channel was measured to be 11.5 µm, see Figure 56, and the actual height of the channel was measured to be 14 µm.

![Image](image_url)

Figure 56: A: A confocal image of fluorescent solution inside several of the microchannels; B: The confocal microscopy measurement results of the actual width of the narrowest channel.
6.3 EXPERIMENTAL SETUP

The micro-fluidic device was placed on the mechanical stage of an optical microscope, Leica DFC490, and was fixed there with tape, shown in Figure 57. A high speed camera, Redlake MotionPro HS-4, was mounted on top of the microscope and connected to a computer with the required software, shown in Figure 58. Experiments were monitored with the computer screen placed next to the microscope. The glass capillaries of the device were connected to a syringe pump via tubing with connectors from Upchurch Scientific. The syringe pump, Harvard PHD 22/2000, was equipped with infuse/withdraw configuration, shown in Figure 58. A container with the cell medium was placed at the outlet of the glass capillaries, as shown in Figure 57. The cells were introduced into the device by suction.

Figure 57: Part of the experimental setup, showing the micro-fluidic device placed in the optical microscope.
Figure 58: The Microscope, the high speed camera and the syringe pump used for experiments.

The complete experimental setup is shown in Figure 59.

Figure 59: The entire experimental setup.
6.4 EXPERIMENTAL PROCEDURE

The experimental procedure, which we reached after a number of optimization loops with trials using various conditions, is explained below step by step:

1- a) BSA coating: The device is filled by flushing 1% w/v (weight per volume) Bovine Serum Albumin (BSA) solution through the device for 2 hours with 1 µL/min. BSA coats the surfaces of the channels and prevents any other protein adsorption, i.e. it prevents cell sticking. The BSA solution is filtered with micropores having diameter of 0.22 µm to prevent any possible blockage of the channels due to BSA crystals or contaminations in the solution. b) Vascular Cell Adhesion Molecule-1 (VCAM-1) coating: To coat the channels with VCAM-1, we make use of electrostatically binding of VCAM-1 (-) to Poly-D-Lysine (+). Firstly, the device is filled by flushing 0.5 mg/mL Poly-D-Lysine in NaBorate buffer (pH 8.0) for 30 min. The Poly-D-Lysine solution has to be filtered with micropores having diameter of 0.22 µm to prevent any possible blockage of the channels due to contaminations in the solution. Then 10 µg/mL VCAM-1 solution is flushed through the device for 1 hour with 0.1 µL/min. VCAM-1 solution is not filtered like BSA and Poly-D-Lysine solutions. It has to be centrifuged by 10000 rpm for 10 minutes to let possible large particles sediment. This procedure is chosen to prevent a decrease in protein concentration with filtering.

2- Cells are cultured and treated at Philips Life Science Facilities, as described in section 6.1. THP-1 cells are used for the experiments. These are cells from a human acute monocytic leukemia cell line. The cell culture medium is replaced with PBS/0.5% w/v BSA/ 2 mM EDTA solution. Phosphate buffered saline (PBS) is a salty solution containing sodium chloride, sodium phosphate, and (in some formulations) potassium chloride and potassium phosphate. The buffer helps to maintain a constant pH. Ethylene diamine tetraacetic acid (EDTA) is used as an agent that binds to calcium and magnesium ions and inhibits pseudopod formation and ensures that the cells maintain essentially spherical. The concentration of the cells used in the device is between 5·10^5 and 6·10^5 cells/mL.

3- The cells are introduced by suction from a container at the inlet of the device with the flow rate of the syringe pump of 1 µL/min for the one-channel device, and 5 µL/min for the capillary network device. With lower flow rates sedimentation and sticking of the cells in the glass capillaries of the device tended to occur, leading to almost zero cell entry in the device.

4- Movies are made by using the high speed camera described earlier, with 2000 frames per second and with 150-250 µs exposure time to prevent image blurring.

5- Since the cells also sediment in the container at the outlet, pipetting in the container up and down every 5 minutes helps to maintain a homogenous cell solution.

6- The experiments have to be conducted within 2 hours after taking the cells of their medium, since the viability of cells is expected to be compromised after 2 hours.

7- After completion of the experiment, the cleaning procedure for the device is as follows: Flushing through the device:
- Distilled water for 1 hour
- 1M HCl solution for 30 minutes
- Distilled water for 1 hour
- 1M NaOH solution for 30 minutes
This procedure is performed twice.
6.5 IMAGE ANALYSIS METHOD

We developed a dedicated MATLAB routine to analyze the movies taken by the high speed camera during experiments. The local and the average velocities, as well as the sizes of the cells introduced into the micro-fluidic device can be determined using this routine. The MATLAB code is given in Appendix IV. The analysis method is explained below.

A region of interest is selected to determine the pixel size (μm/pixel) in the movie as indicated in Figure 60 and the boundaries of the channels are detected. Since the actual size and the number of pixels in the region of interest are known, the pixel size can be simply calculated. In our experiments, the pixel size was around 0.6 μm.

![Image of pixel size calculation](image)

**Figure 60:** The pixel size calculation using MATLAB.

The next step is to select a region of interest to carry out image analysis. Mostly these are the channels through which cells transit. The region of interest is cropped from each frame of the movie, see Figure 61 A. A background subtraction method is applied to the cropped part of each frame to identify moving cells, see Figure 61 B. The background is the median of frames over time. After subtraction of the background from each frame, the pixel values are integrated along the width of the region of interest for each frame, see Figure 61 C.
The integration of the pixel intensity values results in a typical signal, given by the blue curve in Figure 62 A. The minima of the signal define the position of the cell boundaries. The distance between them therefore defines the cell size in that frame. The position of the cell is defined by the average of cell boundary positions. Since both position and frame rate are known, the velocity of the cell can be simply computed. When the resulting signal after the background subtraction for each frame defines one row of a matrix and this matrix is displayed as an image, then the movement of the cell boundaries can be seen very well for each frame, shown in Figure 62 B. The image represents the position of the cell in the region of interest on the horizontal axes and time on the vertical axis. The inverse of the slope of the line relates directly to the velocity of the cell and its width to the size of the cell. By the use of our dedicated MATLAB routine, the local and the average velocities, as well as the sizes of the cells through a region can be easily calculated with the use of this analysis method, see Figure 63. This figure also shows that the slope of the line is constant which means that the cells have the same velocity throughout the channel.
Figure 62: A: The integration of the pixel intensity values results in a typical signal; B: The movement of the cell boundaries for each frame through a defined region of interest. The horizontal axis represents the position of the cell and the vertical axis represents time (frame number). The inverse of the slope of the line relates directly to the velocity of the cell and its width to the size of the cell.

Figure 63: The movement of two cells with different sizes through a defined region of interest.
7. CONCLUSIONS

The overall objective of this work was to use the micro-fluidic devices to study the effect of atherosclerosis on the mechanical properties of circulating monocytes. More specifically, our aim was to establish whether any effect in cell velocity occurs and if this can be detected using the micro-fluidic devices.

We studied the effect of atherosclerosis on the mechanical properties of circulating monocytes using two types of micro-fluidic devices with narrow channels: a so-called one-channel design that has just one channel that is narrower than the diameter of the monocytes, and a capillary network device having a number of parallel narrow channels. The monocytes were flushed through the device, and were physically deformed in the narrow channels by interaction with the walls. The deformation and speed of the cells were monitored using a (high speed) camera and analyzed using dedicated image analysis software. We mimicked a number of processes that take place during the development of atherosclerosis by treating the monocytes (THP-1 cells in our case) with Low Density Lipoprotein (LDL) and the inflammatory agent Phorbol 12-myristate 13-acetate (PMA). In addition, the channel walls were coated with the vascular adhesion molecule-1 (VCAM-1) to influence the cell-wall interaction. We used a semi-empirical model to understand our experimental results.

The velocities of the cells in the micro-fluidic one-channel device were successfully determined. None of the treatments mimicking processes in atherosclerosis, though, resulted in a significant change in cell velocities in our device, even though we observed changes in morphology, as well as a change in expression of adhesion and activation markers (for the LDL and the PMA treatment). The experiments with the capillary network device were hampered by cell clogging, which we did not encounter in the one-channel device, probably due to the more gradual converging part of the latter design. The cell clogging lead to large variations in cell velocity, which made the capillary network device unsuitable for the reliable study of the mechanical properties of cells.

We used the semi-empirical model of Lee & Fung\textsuperscript{[33]} to understand the cell velocities observed in the one-channel device. Even though the model contains course approximations, the agreement between the model prediction and the measured data was good. An important conclusion of the model analysis is, however, that our measurements are not sufficiently sensitive to distinguish between effective elastic moduli of cells over a range of 2 orders of magnitude (from 0.1 to 10 kPa). This means that, even though the treatments could have lead to a change in mechanical properties, this would not show up in the measurements significantly, at least for our particular design and measurement conditions.

The device and the experimental conditions need to be optimized to increase the sensitivity to mechanical changes. The channel cross section should be decreased, the flow rate should be decreased, or the number of parallel channels should be increased. All these measures, though, will be bounded by practical limits: manufacturability, image...
analysis resolution, cell inflow limitations, syringe pump resolution, and cell blockage effects.

To understand the observed phenomena in more detail, and to capture sufficiently the complexity of the cell, a numerical approach is needed. This was beyond the scope of our work, and may be subject of a continuation of this work.
8. RECOMMENDATIONS

- For our current approach, the device and the experimental conditions can be optimized to increase the sensitivity to mechanical changes of the cells. In particular, the channel cross section should be decreased, the flow rate should be decreased, and the number of parallel channels should be increased. It should be noted, though, that all these measures will be bounded by practical limits: manufacturability, image analysis resolution, cell inflow limitations, syringe pump resolution, and cell blockage effects. The latter can be minimized by using a gradual contraction from wide to narrow channels instead of the rather sudden contraction in our capillary network device.

- During the optimization of the method, it is necessary to have available cells with different mechanical properties, preferably by modifying the cytoskeleton in a controlled way. For white blood cells, which have a rich actin cortex, a treatment with cytochalasin D can be used. This disrupts the actin polymerization which has a softening effect.

- Fluorescent labeling of the cell cytoskeleton can give us additional information about the contribution of individual components of the cytoskeleton to the deformation. The components of can be individually labeled as illustrated in Figure 64.

![Figure 64: A mouse NIH3T3 fibroblast cell and stained for DNA (blue) and the major cytoskeletal filaments actin (red) and alpha-tubulin (green)\(^1\).](image)

- Next to the transit time measurement, other characteristics may be measured to assess changes in mechanical properties of the cell. One particular example that can be directly applied in our devices is the shape recovery time after the cell’s exit from the narrow channel.

- To efficiently design the device but especially to understand the observed phenomena, more advanced models are needed. To be able to capture sufficiently the complexity of the cell, a numerical approach is necessary. This was beyond the scope of our work. The basic ingredients of the model should be the
viscoelastic properties both of the membrane and of the cytoplasm, as well as the full interaction between the fluid and the cell including surface tension, and the interaction between the cell and the wall. A further refinement of the model would be to explicitly model the cell’s microstructure.

- The most promising applications of the mechanical cell characterization method will be for those diseases that cause a major morphological change of the cells. This happens for cancer, in our case leukemia which affects the white blood cells, and malaria, affecting red blood cells. Rather than providing a direct diagnosis method, mechanical characterization could provide a pre-screening method as a step before additional clinical tests. Another possibility is to distinguish between the stages of the disease.
- After optimization with model cells and cell lines, the use of cells from patients is required to reliably decide whether the method can be applied to a disease.
ACKNOWLEDGEMENTS

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REFERENCES


APPENDIX I: CLASSIFICATION OF CIRCULATING CELLS

White blood cells, or leukocytes, are cells of the immune system defending the body against both infectious disease and foreign materials. Several different and diverse types of leukocytes exist, but they are all produced and derived from a multipotent cell in the bone marrow known as a hematopoietic stem cell, from which all circulating cells are derived. A complete classification of circulating cells is shown in Figure 65. A full description can be found in [44].
Figure 65: Classification of circulating cells.
APPENDIX II: EXISTING TECHNIQUES FOR MEASURING MECHANICAL PROPERTIES OF CELLS

A wide variety of experimental methods have been used to extract the mechanical properties of cells, see Figure 66.

In Atomic Force Microscopy (AFM), magnetic twisting cytometry and cytoindentation, a surface portion of an adherent cell is mechanically probed using forces on the order of $10^{-12}$ - $10^{-6}$ N and displacements smaller than 1 nm.

In the AFM method, local deformation is induced on the cell surface with a flexible cantilever driven at a constant piezo extension rate with respect to the cell. The deflection of the cantilever is recorded by reflecting a laser of the cantilever onto a split photodiode. Rosenbluth et al. used a linear elastic model based on Hertzian mechanics to determine the apparent stiffness of human myeloid leukemia (AML- HL60 cell line), human lymphoid leukemia (ALL- Jurkat cell line) and neutrophils from AFM.


Hertzian mechanics studies the contact between two linear elastic bodies at small strains. The theory gives the relationship between the force and the displacement when an elastic body is indented with a rigid sphere, in terms of the elastic properties of the body.
measurements. HL60 are neutrophilic human promyelocytic leukemia cells, and Jurkat cells are human (immortalized) T lymphocytes (see Appendix I).

In the magnetic twisting cytometry method, a torque is applied to a magnetic microbead bound to the cell surface which causes cell deformation, see Figure 67. Responses to step and sinusoidal loadings can be studied, so that viscoelastic properties of a single, isolated cell can be determined over a wide range of time scales and forcing amplitudes\textsuperscript{17}.

![Figure 67: Scanning electron micrograph of a red blood cell with a magnetic bead bound to the surface\textsuperscript{17}.](image)

With the laser/optical tweezers method, the microplate stretcher, the microfabricated post array detector, and micropipette aspiration forces over the range of $10^{-12}$–$10^{-6}$ N can be induced on the whole cell while sub-micrometer displacements are monitored optically.

In the optical tweezers method a high refractive index dielectric bead is attached to the surface of a cell. The passage of a laser beam through that bead results in a change in momentum of the photons of the beam. The resulting change in momentum exerts a force on the bead and pushes it towards the focal point of the laser beam which causes a displacement of the cell membrane. The optical tweezers method can be used to stretch a single, isolated cell directly in one or more directions by trapping beads that are strategically attached to the cell surface\textsuperscript{18}.

In the microplate stretcher method, two glass plates are attached to the cell surfaces which can induce force or displacement controlled extensional or shear deformation, see Figure 68.

![Figure 68: Microplates for manipulation and mechanical perturbation of individual cells\textsuperscript{19}.](image)
In the microfabricated post array detector method, a cell is tethered to the surfaces of a substrate patterned with microfabricated flexible cantilevers. Their deflection can be used to calibrate the local force of adhesion. Figure 69 shows an example of a cell attached to flexible micro-posts.

Figure 69: Scanning electron micrograph of a muscle cell attached to an array of microfabricated posts that was uniformly coated with fibronectin\textsuperscript{20}.

In the micropipette aspiration method a portion of a cell or the whole cell is aspirated through a micropipette by applying suction, see Figure 70. The micropipette diameter is smaller than the cell diameter. During the course of the experiment, the deformation of the cell is recorded by means of optical microscopy, which provides the elastic and viscoelastic responses of the cell when an appropriate analysis is applied. The experiment consists of three basic stages. First, the cell is aspirated into the micropipette partially. After exceeding a threshold pressure, the cell is sucked into the pipette completely. This threshold is created by the surface tension of the cell. Finally, after a certain holding time, the cell leaves the pipette and recovery is observed. All three stages give typical cell deformation behavior, which depends on the pipette diameter, holding time, and applied pressure\textsuperscript{21}. Micropipette aspiration experiments are widely used to fit the mechanical models to describe the behavior of leukocytes observed in such experiments.

Figure 71: Micropipette aspiration of a cell\textsuperscript{21}.

The shear flow method is a method with which the biomechanical response of a cell population can be analyzed by monitoring the shear resistance of cells to shear flow\textsuperscript{45}. In
the *substrate stretcher* method, the cells are attached on polymeric substrates with which the mechanics of cell spreading, deformation and migration in response to imposed deformation can be extracted[1].

Many of the techniques explained above are performed on a per-cell basis. Therefore, they require single-cell handling which can make the used technique very slow (several cells per hour). For a proper diagnosis, often, statistics on a large number of cells is needed to achieve adequate sensitivity and specificity. Moreover, for some diseases it is necessary that it is diagnosed quickly, so that the proper treatment can be received by the patient in time. The above methods do provide the accuracy and control needed in basic scientific research but do not provide the speed and automation to be exploited commercially for high-throughput diagnostic tests.

One method that can be used for high-throughput screening of cells is the *optical stretcher method[^46]*. This stretcher is a coaxially aligned dual-beam laser tweezers system shown in Figure 72. When a dielectric material, for example a biological cell, is placed between two opposed laser beams, the total force acting on the object is zero but the surface forces are additive, thus leading to a stretching force acting on the cell along the axis of the beams. This can be performed within a micro-fluidic channel without any contact between the cell and the walls. The applied forces are in the range of $10^{-10} - 10^{-12}$ N. This method has been shown to be able to successfully distinguish between non-malignant and malignant human breast cancer cells. Disadvantages of the optical stretcher concept are that a heavy and expensive laser is needed for the trapping. Moreover, the claimed throughput (~1 cell/s) is far exceeded by the throughput obtained by staining-based flow cytometry (>1000 cells/s). Finally, the cells may be damaged by the radiation of the laser beam.

![Figure 72: The optical stretcher: Cells flowing through a micro-fluidic channel can be serially trapped (A) and deformed (B) with two counter-propagating divergent laser beams[^46].](image)

One other method that can be used for high-throughput screening of mechanical properties of cells is *micro-fluidics*. One advantage of micro-fluidics is that it offers opportunities to study mechanical properties of a single, non-isolated cell in a capillary-like microenvironment, under physiological conditions. A micro-fluidic device contains sub-millimeter channels, down to sizes of just micrometers, in which liquids and (bio-)
molecules can be manipulated. Other advantages of micro-fluidic systems are easy fabrication and rapid prototyping. Shelby et al.\textsuperscript{[23]} monitored the deformability and the shape recovery time of a single uninfected and malaria infected red blood cell by flowing through capillary-like channels in such a micro-fluidic chip. Infected red blood cells showed a lack of deformability and longer recovery time. Rosenbluth et al.\textsuperscript{[17]} developed a micro-fluidic device that trifurcates into two wide by-pass channels and a network of bifurcating channels which split into 64 parallel capillary-like microchannels, resulting in a biophysical flow cytometer device that resembles the micro-capillary network, see Figure 73. The central microchannels are 5.89±0.08 μm wide by 13.3 μm tall by 130 μm long. Rosenbluth et al. measured single-cell transit times of blood cell populations passing through the capillary network. Samples from an AML (Acute myeloid leukemia) patient with leukostasis\textsuperscript{22} symptoms (AML2), an AML patient without leukostasis symptoms (AML1), and two ALL (Acute lymphoblastic leukemia) patients without leukostasis symptoms, as well as neutrophils and red blood cells (RBC’s) from healthy volunteers were used. The results will be discussed in Appendix III.

**Figure 73:** The biophysical flow cytometer of Rosenbluth et al.\textsuperscript{[17]} A: Blood cells were loaded into a syringe and flowed into the device at a constant flow rate. The cytometry device trifurcates into two wide by-pass channels and a network of bifurcating channels which split into 64 parallel capillary-like microchannels. Scale bar 1 mm; B: 16 of these microchannels are shown here. Scale bar 100 μm; C: 75th percentile of transit times of all cells.

\textsuperscript{22} Leukostatis is an abnormal intravascular leukocyte aggregation and clumping often seen in leukaemia patients.
APPENDIX III: DISEASES ASSOCIATED WITH ALTED MECHANICAL PROPERTIES OF CELLS

Upon performing a literature search, a number of diseases were found to be associated with a change in the mechanical properties of cells, in particular their stiffness. These form potential applications for the high-throughput measurements of cell elasticity. They can be grouped in different categories:

- Oncology
- Cardiology and hematology
- Infectious diseases

ONCOLOGY

Several studies have shown clear differences in elasticity between healthy cells and cancerous cells.

Breast cancer

In optical stretcher measurements\cite{47}, it was shown, that cancerous and metastatic cells are significantly more compliant than healthy cells\cite{46}. Furthermore, a healthy epithelial cell\textsuperscript{24} line was compared with comparable cancerous and metastatic\textsuperscript{25} breast epithelial cell lines. The optical deformability measurements of breast epithelial cell lines are shown in Figure 74. It was proposed to use similar methods to analyze cells taken from a needle biopsy of early breast tumors.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure74.png}
\caption{Optical deformability of normal, cancerous, and metastatic breast epithelial cells, taken from MCF10, MCF7, and modMCF7 cell lines, respectively\cite{46}.}
\end{figure}

\begin{thebibliography}{9}
\bibitem{47} This appendix is based upon R.G.H. van Leeuwen, M. Yavuz, “Microfluidics solutions to cell mechanics measurements; applications in cell diagnostics,” Philips Technical Note: PR-TN 2008/00317, 2008.
\bibitem{46} Epithelial cells are the cells that cover a body surface or line a body cavity.
\bibitem{25} When a cancer spreads (metastasizes) from its original site to another area of the body, it is termed metastatic cancer.
\end{thebibliography}
**Lung, breast and pancreas cancer cells in pleural fluid**

An Atomic Force Microscopy (AFM) study showed that the metastatic cancer cells, taken from the pleural fluid\(^{26}\) of lung, breast and pancreas cancer patients, are more than 70% more compliant than the healthy cells lining the body (pleural) cavity from which the fluid was taken\(^{48}\). Histograms of these results are shown in Figure 75. Although the differences between cancer cells and healthy cells are significant, no significant differences could be observed between different cancer types. Moreover, for all patients, the cancer had progressed into the metastatic stage. Hence, this study does not support using elasticity measurements for the diagnosis of type of cancer using cells taken from the pleural fluid. A potential application here is patient monitoring during therapy. Pleural needle biopsy can be performed and a cell-mechanics based method can be applied to the acquired cells within the pleural fluid.

![Histograms of the Young’s modulus E for samples collected from patients with suspected metastatic cancer. a: Histogram collected from seven different clinical samples (tumor, n=40; normal, n=48); b: Tumor data and Gaussian fit; c: Normal cell data with log-normal fit\(^{48}\).](image)

**Leukemia**

As mentioned earlier in Appendix II, Rosenbluth et al.\(^{5}\) used AFM in combination with a linear elastic model based on Hertzian mechanics to determine the apparent stiffness of human myeloid leukemia (AML- HL60 cell line), human lymphoid leukemia (ALL- Jurkat cell line) and neutrophils. HL60 cells were measured to be a factor of 18 times stiffer than Jurkat cells and six times stiffer than human neutrophils on average. (\(E_e = 855 \pm 670\) Pa for HL60 cells, \(E_e = 48 \pm 35\) Pa for Jurkat cells, \(E_e = 156 \pm 87\) Pa for neutrophils), see Figure 76\(^{5}\). This shows that leukemic cells from different cell lines have a different stiffness. This difference is consistent with the fact that AML leads more often to leukostasis\(^{27}\) than ALL.

\(^{26}\)The pleural fluid is the fluid in the body cavity that surrounds the lungs.

\(^{27}\)Abnormal intravascular leukocyte aggregation and clumping often seen in leukaemia patients.
Rosenbluth et al. measured single-cell transit times of blood cell populations obtained from leukemia patients (rather than from cell lines) passing through the capillary network shown earlier in Figure 73. Samples from an AML (Acute myeloid leukemia) patient with leukostasis symptoms (AML2), a AML patient without leukostasis symptoms (AML1), and two ALL (Acute lymphoblastic leukemia) patients without leukostasis symptoms, as well as neutrophils and red blood cells (RBC’s) from healthy volunteers were used. The leukostasis-symptomatic AML patient sample cells took significantly longer to deform into the microchannels than both the leukostasis-asymptomatic AML patient and the ALL patients, as well as the normal neutrophils and red blood cells. The histograms of the leukemia samples show that the majority of the cells of all leukemia samples deformed into the microchannels in less than one second. Median transit times of all blood cells were remarkably similar, ranging between 0.13–0.50 s, see Table 5. The leukostasis-symptomatic sample, however, had an increased distribution of slow transit time outliers. This behavior becomes more apparent when looking at the number of cells that transited in greater than four seconds, and at the 75th percentiles of transit time which is over nine times higher in the leukostasis-symptomatic sample than the leukostasis-asymptomatic samples, see Table 5 and Figure 77.

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**Figure 76: Results of AFM stiffness measurements of human myeloid leukemia (AML- HL60 cell line), human lymphoid leukemia (ALL- Jurkat cell line) and neutrophils**

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28 Order statistics provide a way of estimating proportions of the data that should fall above and below a given value, called a percentile. The \( p \)th percentile is a value, \( Y_{(p)} \), such that at most \( (p)\% \) of the measurements are less than this value and at most \( (100- p)\% \) are greater. The 50th percentile is called the median. Percentiles split a set of ordered data into hundredths. For example, 75% of the data should fall below the 75th percentile.
Table 5: Summary of transit time and microchannel occlusion data obtained by Rosenbluth et al\[7\].

<table>
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<tr>
<th>Cell type</th>
<th>n</th>
<th>25%</th>
<th>50%</th>
<th>75%</th>
<th>Pass through %</th>
</tr>
</thead>
<tbody>
<tr>
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<td>196</td>
<td>0.13</td>
<td>0.13</td>
<td>0.23</td>
<td>100.0</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>254</td>
<td>0.23</td>
<td>0.33</td>
<td>0.53</td>
<td>98.4</td>
</tr>
<tr>
<td>ALL1</td>
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<td>0.30</td>
<td>0.57</td>
<td>95.4</td>
</tr>
<tr>
<td>ALL2</td>
<td>128</td>
<td>0.13</td>
<td>0.23</td>
<td>0.40</td>
<td>94.5</td>
</tr>
<tr>
<td>AML1</td>
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<td>0.20</td>
<td>0.83</td>
<td>91.5</td>
</tr>
<tr>
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<td>0.50</td>
<td>7.75</td>
<td>81.2</td>
</tr>
</tbody>
</table>

Figure 77: The transit times of four patient leukemia cells, measured by Rosenbluth et al. \[7\]. The median transit times are very similar; however the AML2 cells have an increased number of long transit times far away from the average.

**Circulating tumor cells**

A complication with the application of cell mechanics measurements in the diagnosis of cancer is that mostly, cancer cells are embedded in a tumor (with the exception of cancer cells in blood cancers). Isolating individual cells from the tumor may alter their elasticity. It has been shown that in the presence of a metastasing tumor, circulating cancer cells (cancerous cells in the blood stream, originating from the tumor) are present in very low numbers in the circulation (5 in 10 mL of blood)\[49\]. These circulating tumor cells (CTC’s) only occur once a cancer has progressed in the metastasis stage. The molecular analysis of CTC’s is expected to provide additional information on the metastatic potential of the tumor, as well as the response to systemic targeted therapy. Also following treatment these cells may provide an excellent tumor cell source to monitor the response to treatment as well as to predict therapy response in the case of recurrence or metastasis. However for this molecular analysis (DNA, RNA protein) the cells first have to be obtained. Currently only in some cases antibody-mediated capture of CTC’s that carry a
A tumor-specific antigen is possible. Unless the antigen is located on the cell membrane, the cells need to be fixed which makes them unsuitable for specific molecular (protein, mRNA) analyses. Also, antigen expression may change with the evolution of the tumor, allowing them to escape detection. Thus, isolation of unlabelled tumor cells would be a big improvement. Rather than for direct diagnostic purposes, a cell isolation/selection method based on the measurement of the mechanical properties of circulating cells could provide a way of isolating CTC’s. Integrated in a micro-fluidic device, this could lead to a high-throughput cell isolation method that does not require labeling.

**Oral cancer**

After the operative removal of an oral cancer tumor, residual cancer cells can remain which can eventually grow into metastasis. To check whether all cancer cells have been removed from the tumor site, cell smears could be analyzed by measuring the cell mechanics of individual cells[49].

**CARDIOLOGY/HEMATOLOGY**

A few diseases related with cardiology/hematology may be associated with a change in the mechanical properties of white blood cells (WBC’s). WBC’s are twice larger in volume per cell and 2000 times more viscous than red blood cells, resulting in low deformability and slow transit through the capillary network having a diameter in the range of 4-10 μm. Activation of white blood cells, e.g. by peptide chemoattractants, renders them stiffer due to polymerization of cortical actin and by the projection of pseudopods with crosslinked actin. Furthermore, the cells become more adhesive. These properties combined lead to a high probability for activated white blood cells to become trapped in the capillaries, a phenomenon that is termed leukostasis[6][50] and often observed in patients with leukaemia.

A difficulty with the analysis of white blood cells is that they constitute a small fraction of whole blood (1%). For a proper analysis, often, red blood cells must be removed prior to cell mechanics measurements.

**Ischemia**

Ischemia is a restriction in blood supply with resultant damage or dysfunction of the tissue. White blood cell activation may play a role in ischemia with resultant altered adhesive and mechanical properties which increase their tendency to plug the capillaries and may result in an ischemia vicious circle: white blood cell trapping and activation, endothelial damage with release of oxygen free radicals and digestive enzymes, and further activation and trapping of new cells[51]. Such mechanisms have been observed in leg ischemia[8][9].
**Acute myocardial infarction**

Mononuclear cells (monocytes and lymphocytes) after acute myocardial infarction (AMI) show an increased resistance to flow through filters with 8 μm micropores. This may play a role in the blocking of the capillaries that leads to the infarction and/or it may contribute to the tissue ischemia after infarction\(^{[52]}\). This increased resistance, and an increase in the resistance to flow of granulocytes within 3 days from the infarction was not observed for a control group with chest pain but no AMI. Hence, it was claimed that the deformability of WBC’s can be an indicator for whether an infarction has taken place for patients with chest pain. For patients aged >60 years, the described effects were not observed.

**Atherosclerosis**

Atherosclerosis is a chronic inflammatory condition which occurs within discrete regions of the arterial walls. It may be another disease that could be associated with mechanical properties of white blood cells, in particular of monocytes which play a key role in the development of atherosclerosis\(^{[12]}\). The function of monocytes is to protect tissue from foreign substances. In response to an inflammation in the tissue, monocytes are activated, leave the bloodstream via the inner layer of the blood vessel, the endothelium, and migrate into the tissue. This process is shown in Figure 2A. After differentiation into so-called macrophages they attack and digest foreign microorganisms and dead cell debris. Adhesion molecules are involved in the adhesion of monocytes to endothelial cells and in their migration into the subendothelial space. Monocytes come in random contact with endothelial cells, and the adhesion molecule *E-selectin* expressed by endothelial cells causes a slowing down of monocytes flowing in the active blood stream. As a consequence they roll over the endothelium. Then, monocytes attach firmly with integrins\(^{29}\) on their surface (VLA-4 and Mac-1) to endothelial adhesion receptors, i.e. vascular cell adhesion molecule-1 (VCAM-1) or intercellular adhesion molecule-1 (ICAM-1). Once monocytes are tightly bound, they migrate into the subendothelial space in response to monocyte chemotactic protein-1 (MCP-1) expressed by endothelial cells\(^{[14]}\).

\(^{29}\)Integrins are cell surface receptors that play a role in the attachment of cells to other cells.
In the development of atherosclerosis, the process of transmigration of the monocytes into the tissue is modified, as shown in Figure 2B.

The disease begins when the inner layer, the endothelium, is damaged. There can be various causes of this damage, such as high cholesterol level, smoking, hypertension, diabetes, and obesity\textsuperscript{[15][16]}. As a result of the damage of the endothelium, its permeability for Low Density Lipoprotein (LDL)\textsuperscript{30} increases, so that this lipoprotein accumulates in the subendothelial space. Having arrived in the subendothelial space, LDL undergoes oxidation. The accumulation of oxidized LDL activates endothelial cells to express monocyte chemotactic protein 1 (MCP-1), which attracts monocytes from the bloodstream into the subendothelial space. There, the monocytes differentiate into macrophages. The macrophages release a variety of chemicals, including cytokines\textsuperscript{31}, in order to activate endothelial cells to express adhesion molecules, i.e. VCAM-1 and ICAM-1, which bind to integrins on monocyte surfaces, i.e. VLA-4 and Mac-1, respectively, making them available for recruitment into the subendothelial space by MCP-1. Subsequently, by taking up oxidized LDL, the macrophages slowly turn into large so called "foam cells". This loop of adhesion, migration, differentiating, adhesion molecule expression, uptake of oxidized LDL repeats\textsuperscript{[14]}. This is the beginning of atherosclerotic plaque formation which continues by the accumulation of fat-laden foam cells, smooth muscle cells and other materials, to form a patchy deposit called atherosclerotic plaque, see Figure 3. As this grows, it thickens the artery wall and bulges into the artery. This may narrow or block an artery, reducing or stopping blood flow. Since the arteries supply the myocardium (the muscle of the heart) with oxygen and nutrients, this results in Coronary Artery Disease (CAD) (or atherosclerotic heart disease) i.e. a lack of oxygen supply to heart tissues (ischemia) which can lead to death of heart tissue and over time to heart failure.

\textsuperscript{30} LDL is a lipoprotein that is present in the bloodstream at optimal levels between 100 and 129 mg/dl; its normal function is to carry fats and cholesterol from the liver to peripheral tissues.

\textsuperscript{31} Cytokines are a category of signaling molecules that, like hormones, are used extensively in cellular communication. They are proteins or glycoproteins.
Figure 79: Development of atherosclerosis: The presence of cellular and lipid deposits on the arterial walls begins a chain of complex processes \(^{[16]}\).

No studies have been published on the possible effect of atherosclerosis on the mechanical properties of monocytes. It could be speculated, that the activation of the monocytes by cell adhesion molecules changes their morphology and therefore their mechanical properties. Also, the presence of native LDL \(^{[32]}\) has been shown to change the expression by monocytes of a number adhesion and activation markers, which may lead to changes in their adhesive and mechanical properties.

On the other hand, the activation may not be specific for atherosclerosis, since many diseases may activate monocytes by a response of the immune system. However if we have some previous knowledge about the presence of atherosclerosis, we may differentiate between the stages of the disease through a measurement of the mechanical properties of the monocytes and make a comparison of the change of deformability.

**Diabetes**

Blood of patients suffering from diabetes type II was shown to flow significantly slower through an array of parallel, 6 \(\mu\)m-wide microchannels than blood of control subjects and blood with white blood cells removed\(^{[53]}\). Therefore, it was claimed that white blood cells have a reduced deformability for these patients. This reduced deformability could lead to vascular disorders of diabetic patients. Some snapshots of this study are shown in Figure 80.

\(^{32}\) Native LDL means not oxidized LDL.
Another study\textsuperscript{[10]} using filters with 5 μm pores showed that white blood cell (WBC) suspension of diabetes (Type I and II\textsuperscript{33}) patients had a reduced filterability when compared to those of a control group without diabetes. Furthermore, WBC suspensions of patients with Type I diabetes showed an increase in the filter clogging compared to WBC suspensions of patients with Type II diabetes. WBC suspensions of diabetes patients suffering from diabetic retinopathy\textsuperscript{34} also showed an increase in the filter clogging compared to those from patients without retinopathy.

**INFECTION DISEASES**

**Malaria**

Red blood cells (RBC’s) of patients infected with malaria can contain Plasmodium falciparum parasites in different stages. Optical tweezers\textsuperscript{[3]}, micropipette aspiration\textsuperscript{[4]} and laminar shear flow\textsuperscript{[54]} measurements of malaria-infected red blood cells showed that the cells stiffen as the parasite develops within. A graph summarizing these results is shown in Figure 81.

\textsuperscript{33} Type 1 diabetes mellitus is characterized by loss of the insulin-producing beta cells in the pancreas, leading to a deficiency of insulin.

Type 2 diabetes mellitus is characterized differently due to insulin resistance or reduced insulin sensitivity, combined with relatively reduced insulin secretion.

\textsuperscript{34} Diabetic retinopathy is the result of microvascular retinal changes.
Figure 81: Effective values of the in-plane shear modulus of the red blood cell (RBC) membrane, estimated from optical tweezers experiments combined with computational simulations of RBC’s infected with Plasmodium falciparum.

In Figure 81 for comparison purposes, shear modulus estimates from micropipette aspiration and laminar shear flow methods are shown, for parasitized RBC’s at different developmental stages of Plasmodium falciparum.

OTHER POTENTIAL APPLICATIONS

Stem cell selection

It has been proposed to use the elastic properties of stem cells to select them from a cell population\(^\text{[46]}\). It has been shown\(^\text{[55]}\) that HL-60 human promyelocytic leukemia cells strengthen their cytoskeleton upon differentiation. This is an indication that stem cells may get stiffer as they differentiate.

Fetal red blood cell recovery

A related application that is proposed is the separation of fetal red blood cells from maternal blood. Maternal blood contains small numbers of fetal red blood cells. Prior to birth, fetal red blood cells still contain a nucleus with DNA. Because of this nucleus, they are likely to have an elasticity that is different from that of the maternal red blood cells. Separating fetal red blood cells enables prenatal genetic screening of the fetus without the need for an amniotic fluid test\(^\text{[35]}\). This test is associated with increased risks to the pregnancy. People have also proposed\(^\text{[56]}\) to use free fetal DNA in maternal blood to perform prenatal genetic tests.

\(^{35}\) Amniotic fluid is the nourishing and protecting liquid contained by the amnion of a pregnant woman and the amnion is a membranous sac that surrounds and protects an embryo.
GENERAL ADVANTAGES OF CELL MECHANICS METHODS

The measurement of the mechanical properties of cells, in particular stiffness, could have a number of advantages over existing techniques used in different stages of a care cycle\(^{[57]}\) (e.g., screening, diagnosis, treatment). In many common methods, e.g. brightfield microscopy and flow cytometry, cells and tissue are stained to create additional contrast. In some cases, (fluorescently) labeled antibodies are used to stain cells for specific molecules present on their surface. Antibodies are very expensive. Moreover, cells stained using antibodies will have an altered functionality which often prevents the cells from being suitable for viable cell-based assays after the staining process. Measuring cell or tissue elasticity can be done without prior staining of cells. This could lead to large savings in costs. Moreover, provided that the studied cells are not affected by an elasticity measurement and not altered biochemically then they can also be used in other assays. For many diseases, diagnosis requires many different assays, and different, often unrelated properties of a cell/patient are assessed. Methods of measuring cell elasticity potentially provide an additional method of diagnosing a disease, and/or to isolate/select cells without any labeling or staining.
APPENDIX I: MATLAB CODE FOR IMAGE ANALYSIS

We developed a dedicated MATLAB routine to analyze the movies taken by the high speed camera during experiments. The local and the average velocities, as well as the sizes of the cells introduced into the micro-fluidic device can be determined using this routine. The MATLAB code is given below:

Loading movie:

% With the code below, the movies are read: each frame is loaded as a % two dimensional matrix. They build together a three dimensional matrix, i.e. the movie. The movies from high speed camera consist of gray scale images.
clc % Clear the command window.
clf % Clear all figures.
clear all
close all
% Loading the movie to be analyzed.
 aviinfo ('ImgA') % ImgA is the name of the movie.
cellmot = aviread ('ImgA');
[height, width, dim] = size ([cellmot(1).cdata]);
numframes = size (cellmot, 2);
gr_cellmot = uint8 (zeros (height, width, numframes)); % allocate memory
% Each frame is loaded as a two dimensional matrix into three dimensional
% matrix corresponding the movie.
for i = 1:1:numframes
    gr_cellmot(:,:,i) = (cellmot(i).cdata);
end
% The frames are flipped if it is needed:
% gr_cellmot1 = uint8 (zeros (height, width, numframes)); % allocate memory
% for i= 1:1:numframes
%    gr_cellmot1(:,:,i)= fliplr(gr_cellmot(:,:,i));
% end
% gr_cellmot=gr_cellmot1;

Calculating pixel size

% The code below finds the pixel size in micrometer. Two points, inbetween which there are at least two(any) border of(any)channels, have to be chosen. The program finds any border inbetween two selected points. Then two borders between which the real distance is known have to be selected by user. The program counts the pixel numbers between the chosen borders. Thus, the pixel size can be calculated.
clear roi;
clear p1; clear p2; clear f1; clear f11; clear f12; clear f2; clear f21;
clear f22; clear x11; clear x12; clear x21; clear x22; clear firstx; clear firstxx;
clear secondx; clear secondxx; clear firsty; clear secondy; clear smoothed; clear kernel;
clear minp1; clear minp1x; clear minp1y; clear minp2; clear minp2x; clear minp2y;
clear currentIm; clear intProfile; clear x; clear kernel; clear kWidth;
clear firstbordercol; clear secondbordercol; clear co; clear ro; clear
col; clear row;
clear cellmot; clear sizeL; clear fromRow; clear toRow; clear
pixellength; clear reallength; clear pixsize

% Select two points (see above).
% Then select two (any) borders of (any) channel in-between the selected
% points and give the real distance between them to the program.
currentIm = gr_cellmot(:,:,1);
figure(1); clf; imshow( currentIm );
figure(1); [co,ro]=ginput(2);
col = round( mean(co) );
fromRow = round( ro(1) );
toRow = round( ro(2) );
intProfile = sum( currentIm( fromRow:toRow,col+-5:5),2 )/11;
sizeL = length(intProfile);

% Illustrate the region inbetween two selected points.
hold on; rectangle( 'Position' ,[col-5,fromRow,10,toRow-fromRow] )
sigma=1.4;
x = floor(-3*sigma):ceil(3*sigma);
kernel = (x.^2-sigma^2)/(2*pi*sigma^6) .* exp( -(x.^2)/(2*sigma^2) );
kWidth = length(kernel);
smoothed = conv( double(intProfile),kernel );
smoothed = smoothed(floor(kWidth/2)+[1:sizeL]);
xx=1:length(intProfile);
figure(2); clf; imagesc( currentIm( fromRow:toRow,col+-10:10) );
colormap(gray); axis off;
hold on; plot(smoothed-min(smoothed(:)));

% Select a point on top of one border in the region of interest and a
% point on top of the second border.
[col row]=ginput(2);
firstbordercol=round(col(1));

% The chosen points may not correspond the borders: The minimum is
% found around the fisst chosen points corresponding the first channel
% borders
minp1=firstbordercol-3;
for i=-2:1:3
  if smoothed(firstbordercol+i)>smoothed(minp1)
    minp1=firstbordercol+i;
  end
end

% Plot the minimum corresponding the first border.
minply=smoothed(minp1);
plot(minply,minply-
min(smoothed(:)),'o','LineWidth',2,'MarkerEdgeColor','r',
'MarkerFaceColor','b',
'MarkerSize',2);

% The minimum is found around the second chosen point corresponding the
% second channel border.
secondbordercol=round(col(2));
minp2=secondbordercol-3;
for i=-2:1:3
  if smoothed(secondbordercol+i)>smoothed(minp2)
    minp2=secondbordercol+i;
  end
end

% Plot the minimum which defines the second border.
minp2y = smoothed(minp2);
plot(minp2, minp2y - min(smoothed(:)), 'o', 'LineWidth', 2, 'MarkerEdgeColor', 'r', ...
    'MarkerFaceColor', 'b', ...
    'MarkerSize', 2);

% Count the number of pixels between the lines
pixellength = abs(minp1 - minp2);
% Get the real distance between the biological cell borders
reallength = input('Give the real length between the borders you have chosen: ');
% Pixel size calculation
pixsize = reallength / pixellength

Choosing a region of interest:

% With the code below, the region(s) of interest is(are) chosen where the cell size and their velocities should be calculated.
clear clf;
clear roi;
clear p1; clear p2; clear f1; clear f11; clear f12; clear f2; clear f21;
clear f22; clear x11; clear x12; clear x21; clear x22; clear firstx;
clear firstxx;
clear secondx; clear secondxx; clear firsty; clear secondy;
clear smoothed; clear kernel
clear minp1; clear minp1x; clear minp1y; clear minp2; clear minp2x; clear minp2y;
clear currentIm; clear intProfile; clear x; clear kernel; clear kWidth;
clear firstbordercol; clear secondbordercol; clear co; clear ro; clear col;
clear row;
clear cellmot; clear sizeL; clear fromRow; clear toRow;
clear roichannel; clear background; clear roichannel1; clear background1;
clear backdiff; clear Med; clear pixel; clear i; clear j; clear k; clear y;
clear sigmaPre; clear kernel; clear sum_ints; clear storedsum_ints;
clear threshold_noise; clear totalnumber; clear total ; clear"threshold; clear imgsqnc
clear Framenumberswithcell; clear numberofframeswithcell; clear borders;
clear endborders; clear Velocityofcell, clear Sizeofcell; clear Middlpoint;
clear sum; clear sumc; clear storedsum_ints; clear beginframe; clear endframe;
clear cellbeginframe; clear cellendframe; clear valley; clear AverageCelldata
clear lastendborders; clear fliplastendborders; clear flipborders;
clear flipendborders; clear deletenoise; clear cellframes
% Showing the first frame of the movie to choose the region of interest.
% Which will be cropped from all the frames of the loaded movie.
figure(1), imshow(gr_cellmot(:,:,1))
% Msgbox('choose your region of interest','help','help');
maskposition = [];
h = imrect(gca, maskposition);
% Saving the coordinates of the region of interest.
roimask = input('copy position of rectangle on the command window(right click your mouse): ');
maskx = roimask(1);
masky = roimask(2);
masklength = roimask(3);
maskwidth = roimask(4);
maskybegin = masky;
numberroi = input('how many regions of interest in total do you have: ');
y = 2;
% Inserting a number of rectangular required to select other regions of interest.
for i = 1:1:numberroi - 1
    h = imrect(gca, [2 y masklength maskwidth]);
y = y + maskwidth;
end
% Other regions of interest should be chosen and their coordinates should be given to the program.
for i = 1:1:numberroi
    roi(i,:) = input(['copy position of ', num2str(i), ' region of interest on the command window: ']);
end

Background subtraction and detection of moving cells in the region of interest:

% The code below finds the background matrix of the region of interest. It consists of the median of each individual pixel (i.e. each cell of each frame-matrix) within the movie. The background matrix is substracted from each frame-matrix. The resultant frame matrix have matrix-cells (pixels) corresponding to the biological cell borders and the noise. The way of the noise reduction is as follows: The resultant frame-matrix, i.e. frame matrix - background matrix, has a few number of matrix-cells corresponding to the biological cell borders, i.e. the resultant matrix cells with a minimum negative value. Other matrix cells correspond to the noise. Thus, the median of the resultant frame-matrix has to correspond to a noise threshold. Subsequently the noise is reduced by ignoring resultant frame-matrix cells which have a value larger than a noise threshold.
clear roichannel; clear background; clear roichannel1; clear background1; clear backdiff; clear Med; clear pixel; clear i; clear j; clear k; clear y; clear sigmaPre; clear kernel; clear sum_ints; clear borders; clear storedsum_ints;
clear threshold_noise; clear totalnumber; clear total; clear threshold; clear imagesc
clear stored_backdiff_med; clear stored_backdiff_medmatrix; clear sigmaPre; clear kernel; clear sum_ints; clear storedsum_ints; clear threshold_noise;
clear totalnumber; clear total; clear threshold; clear imagesc
clear Framenumberswithcell; clear numberofframeswithcell; clear borders; clear endborders;
clear Velocityofcell; clear Sizeofcell; clear Middlepoint
clear sum; clear sumc; clear storedsum_ints; clear beginframe;
clear endframe; clear valley; clear AverageCelldata; clear lastendborders;
clear fliplastendborders; clear flipborders; clear flipendborders; clear deletenoise
% Choose one region of interest
reply = input('which region of interest do you want to analyse: ')
rect = (roi(reply,:),);
roichannel = (zeros(round(rect(4)) + 1, round(rect(3)) + 1, numframes)); % allocate memory
for i = 1:1:numframes
roichannel(:,:,i)= imcrop(gr_cellmot(:,:,i),rect);
end

% The median of each pixel (i.e. each cell of each frame matrix) within
the movie is calculated. They together build a background matrix of the
region of interest where the cell size and their velocities should be
calculated.
[a b c]=size(roichannel);
pixel=(zeros(c,a*b));
y=1;
z=1;
    for j= 1:1:a
        for k= 1:1:b
            for i= 1:1:c
                pixel(y,z)= roichannel(j,k,i);
                y=y+1;
            end
            y=1;
            z=z+1;
        end
        y=1;
    end
background=(zeros(a,b));
Med=median(pixel);
y=1;
    for i= 1:1:a
        for j= 1:1:b
            background(i,j)= Med(1,y);
            y=y+1;
        end
    end
backdiff=(zeros(a,b,c));
roichannel1=(double(roichannel));
background1=(double(background));
% Subtraction of the background matrix from each frame matrix
sigmaPre = .8;   kernel = fspecial('Gaussian',length(floor(-
3*sigmaPre):ceil(3*sigmaPre)),sigmaPre);
for i=1:c
    backdiff(:,:,i) = roichannel1(:,:,i) - background1;
    % The resultant frame-matrix cells with a minimum negative value
correspond to the pixels of the biological cell borders:"gray(background
matrix)is substracted from black(biological cell border)in the frame-
matrix". Apply little Gaussian smoothing (e.g. sigma=1 results in a 7x7
filter)
    backdiff(:,:,i) = imfilter( backdiff(:,:,i),kernel,'same' );
end
% Ignore pixels>0 : since we are interested in the biological cell
borders, i.e. the resultant frame-matrix cells with a minimum negative
% value.
stored_backdiff=(zeros(a,b,c));
for i=1:c
    stored_backdiff(:,:,i)=backdiff(:,:,i);
end
for i=1:c
    for j= 1:1:a
        for k= 1:1:b
            if stored_backdiff(j,k,i)>=0
                stored_backdiff(j,k,i)=NaN;
            end
        end
    end
end
% Noise reduction
stored_backdiff_med=zeros(1,c);
stored_backdiff_medmatrix=(zeros(1,b,c));
for i=1:c
    stored_backdiff_medmatrix(1,:,i)= nanmedian(stored_backdiff(:,:,i));
end
for i=1:c
    stored_backdiff_med(1,i)=
nanmedian(stored_backdiff_medmatrix(:,:,i));
end
for i=1:c
    for j= 1:1:a
        for k= 1:1:b
            if backdiff(j,k,i)>stored_backdiff_med(1,i)
                backdiff(j,k,i)=0;
            end
        end
    end
end

Calculating local and average velocities and sizes of the cells:

% With the code below the right and left cell borders in the x direction
are found in each frame of the movie.
clear sigmaPre;clear kernel;clear sum_ints; clear storedsum_ints;clear
threshold_noise;clear totalnumber;clear total ; clear threshold;clear
imgsqc
clear Framenumberswithcell;clear numberofframeswithcell;clear borders;
clear endborders; clear Velocityofcell, clear Sizeofcell;clear
Middlepoint
clear sum; clear sumc;clear storedsum_ints;clear beginframe; clear
endframe;clear cellbeginframe; clear cellendframe;clear valley; clear
AverageCelldata
clear lastendborders;clear fliplastendborders;clear flipborders; clear
flipendborders;clear deletenoise;clear cellframes
% Sum the matrix-cell values (pixels) over the width (y direction) of
each resultant frame-matrix, i.e. frame matrix - background matrix.
% Each sum is stored as a two dimensional matrix (1,:) of a three
dimensional matrix, i.e. sum_ints matrix
sum_ints= zeros(1,b,c);
sum=0;
[a b c]=size(backdiff);
for i=1:c
    for k= 1:1:b
        for j=1:1:a
            sumc= sum+ backdiff(j,k,i);
            sum=sumc;
        end
        sum_ints(1,k,i)=sum;
        sum=0;
    end
end
% Sum_ints matrix is stored as a two dimensional matrix, i.e.
% Storedsum_ints matrix: Sum of the matrix-cell values of the first frame
% is the first row of the storedsum_ints matrix and of the second frame is
% the second row.
storedsum_ints = (zeros(c,b));
for sc=1:1:c
    storedsum_ints(sc,:)=sum_ints(:,:,sc);
end
% Search for the frames where the biological cell is in the region of
% interest by defining
% a threshold value below which the sum_ints matrix-cell(pixel) may correspond to the biological cell borders. In our
% experiments the threshold is taken -125. (It can change with other high
% speed camera and other frame rates)
threshold=-125;
totalnumber=zeros(1,c);
for i=1:c
    total=0;
    for k= 1:1:b
        if sum_ints(1,k,i)<threshold
            total=total+1;
        end
    end
    totalnumber(i)=total;
end
Framenumberswithcell=(zeros(1,500));
numberofframeswithcell=1;
for i=1:c
    if totalnumber(i)==0
        sum_ints(:,:,i)=0;
    end
    if totalnumber(i)~=0
        Framenumberswithcell(numberofframeswithcell)=i;
        numberofframeswithcell=numberofframeswithcell+1;
    end
end
numberofframeswithcell=numberofframeswithcell-1;
% Find the frames when the cell enter&leaves the region of interest
beginframe=Framenumberswithcell(1); % The begin frame is the frame when
% the cell enters the region of interest.
endframe=Framenumberswithcell(numberofframeswithcell); % The end frame
% is the frame when the cell leaves the region of interest.
% Find a noise threshold: The storedsum_ints matrix-cells, which have
% values lower than -50, may correspond the biological cell borders.
% The median of the storedsum_ints matrix-cells which have a value above -50
% and below 0 correspond to a noise threshold. The storedsum_ints matrix-
% cells which have a value larger than the noise threshold will be
% ignored, i.e. the matrix-cell value is set to 0.
for i=1:c
    for k= 1:1:b
        if storedsum_ints(i,k)==0
            storedsum_ints(i,k)=NaN;
        end
        if storedsum_ints(i,k)<=-50
            storedsum_ints(i,k)=NaN;
        end
    end
end
storedsum_ints_med= (zeros(1,c));

for i=1:c
    storedsum_ints_med(1,i)=nanmedian(storedsum_ints(i,:));
end

% Looking for the frames where both of two borders of the biological cell are in. Ignore the frames where the cell enters & leaves the region of interest.

for m=1:1:(numberofframeswithcell)
    i=Framenumberswithcell(m);
    for k=2:1:b-1
        if sum_ints(1,k,i)>= -55
            sag=0;
            sol=0;
            for j=k:-1:1
                if sum_ints(1,j,i)<-125
                    sag=1;
                    if sag==1
                        for s=k:1:b
                            if sum_ints(1,s,i)<-125
                                sol=1;
                                break;
                            end
                        end
                    end
                    if (sag==0)
                        sum_ints(1,k,i)=0;
                    end
                    if(sag==1) && (sol==0)
                        sum_ints(1,k,i)=0;
                    end
                    if (sag==1) && (sol==1) && sum_ints(1,k,i)==0;
                        sum_ints(1,k,i)=-1;
                    end
                if (sag==0)
                    sum_ints(1,k,i)=0;
                end
            end
        end
    end
end

% Noise reduction: see above.
for i=beginframe:1:endframe
    if sum_ints(1,1,i)>=storedsum_ints_med(1,i)
        sum_ints(1,1,i)=0;
    end
end

for i=beginframe:1:endframe
    if sum_ints(1,b,i)>=storedsum_ints_med(1,i)
        sum_ints(1,b,i)=0;
    end
end

% The sum of the matrix-cell values over the width (y direction) of each resultant frame-matrix is stored as a row of a matrix, i.e. "imgsqnc" matrix.
imgsqnc= (zeros(c,b));
for sc=1:1:c
% It is found how many cells enter the region of interest. The begin and % end frames for each cell, i.e. the frames when the cell enters and leaves the region of interest, respectively, are saved. The cells should not be in the region of interest at the same time!
number=1;
j=2;
Celldata(1,1,number)=Framenumberswithcell(1);
cellframes(1,1,number)=Framenumberswithcell(1);
for i=2:1:numberofframeswithcell
    if Framenumberswithcell(i)-1==Framenumberswithcell(i-1);
        Celldata(j,1,number)=Framenumberswithcell(i);
        j=j+1;
    end
    if Framenumberswithcell(i)-1~=Framenumberswithcell(i-1);
        cellframes(1,2,number)=Framenumberswithcell(i-1);
        number=number+1;
        j=1;
        Celldata(j,1,number)=Framenumberswithcell(i);
        cellframes(1,1,number)=Framenumberswithcell(i);
        j=j+1;
    end
end

for h=1:1:number
    cellbeginframe= cellframes(1,1,h);
    cellendframe=cellframes(1,2,h);
    for j=cellbeginframe:1:cellendframe
        cellin=0;
        for k=1:1:b-1
            if imgsqnc(j,k)==0 && imgsqnc(j,k+1)~=0
                count=1;
                for t=k+1:1:b
                    if (imgsqnc(j,t)~=0)
                        count=count+1;
                    end
                end
                if imgsqnc(j,t)<-125
                    cellin=1;
                end
            end
            if (imgsqnc(j,t)==0) && count<12 && cellin==0
                count=1;
            end
        end
    end
end
cellin=0;
bright;
end

if (imsgsqnc(j,t)==0) && count<12
    count=1;
cellin=0;
bright;
end

if (imsgsqnc(j,t)==0) && count>12 && cellin==0
    count=1;
cellin=0;
bright;
end

if imsgsqnc(j,t)==0 && count>12 && cellin==1;
borders(j,1,h)=k; % which pixel(frame-matrix cell) is beginborder.
borders(j,2,h)=t; % which pixel(frame-matrix cell) is endborder.
count=1;
cellin=0;
bright;
end
end
end
end
end

% Find a matrix cell (pixel) with a minimum negative value, i.e. the minimum (valley) between the left temporary cell border and the middle of the cell borders.
for j=cellbeginframe:1:cellendframe
    if borders(j,1,h)~0
        subs=5;
        for x=1:1:10
            if subs<(borders(j,2,h)-borders(j,1,h))/2);
                valleyfind=-500;
            end
            for u=1:1:1000
                if valleyfind<-200
                    sinir=(borders(j,1,h)+subs);
                    for k= borders(j,1,h):1:sinir
                        ((borders(j,2,h)+borders(j,1,h))/2)
                        if imsgsqnc(j,k)<valleyfind && imsgsqnc(j,k-1)>imsgsqnc(j,k) && imsgsqnc(j,k+1)>imsgsqnc(j,k)
                            valley(j,1,h)=k;
                            valley(j,2,h)=imsgsqnc(j,k);
                            break;
                        end
                        end
                    end
                    if valley(j,1,h)==0
                        valleyfind=valleyfind+10;
                    end
                end
            if valley(j,1,h)==0
                valleyfind=valleyfind+10;
            end
        end
    end
end
subs=subs+5;
end
end
end
if valley(j,1,h)==0
    subs=5;
for x=1:1:10
    if subs<((borders(j,2,h)-borders(j,1,h))/2);
        valleyfind=-200;
    for u=1:1:1000
        if valleyfind<=-100
            sinir=(borders(j,1,h)+subs);
            for k= borders(j,1,h):1:sinir
                %((borders(j,2,h)+borders(j,1,h))/2)
                if imgsqnc(j,k)<valleyfind & & imgsqnc(j,k-1)>imgsqnc(j,k) & & imgsqnc(j,k+1)>imgsqnc(j,k)
                    valley(j,1,h)=k;
                    valley(j,2,h)=imgsqnc(j,k);
                    break;
                end
            end
        end
        if valley(j,1,h)==0
            valleyfind=valleyfind+10;
        end
    end
    if valley(j,1,h)==0
        subs=subs+5;
    end
end
end
if valley(j,1,h)==0
    subs=5;
for x=1:1:10
    if subs<((borders(j,2,h)-borders(j,1,h))/2);
        valleyfind=-100;
    for u=1:1:1000
        if valleyfind<=-40
            sinir=(borders(j,1,h)+subs);
            for k= borders(j,1,h):1:sinir
                %((borders(j,2,h)+borders(j,1,h))/2)
                if imgsqnc(j,k)<valleyfind & & imgsqnc(j,k-1)>imgsqnc(j,k) & & imgsqnc(j,k+1)>imgsqnc(j,k)
                    valley(j,1,h)=k;
                    valley(j,2,h)=imgsqnc(j,k);
                    break;
                end
            end
        end
        if valley(j,1,h)==0
            valleyfind=valleyfind+10;
        end
    end
    if valley(j,1,h)==0
        subs=subs+5;
    end
end
delete_noise(j, 1, h) = valley(j, 1, h);

% Find a matrix cell (pixel) with a minimum negative value, i.e. the minimum between the right temporary cell border and the middle of the cell borders.
for j = cell_begin_frame:1:cell_end_frame
    if borders(j, 1, h) == 0
        subs = 5;
        for x = 1:1:10
            if subs < ((borders(j, 2, h) - borders(j, 1, h)) / 2);
                valley_find = -500;
                for u = 1:1:1000
                    if valley_find < -200
                        sinir = (borders(j, 2, h) - subs);
                        for k = borders(j, 2, h):1:sinir
                            if (borders(j, 2, h) + borders(j, 1, h) / 2)
                                if imgsnc(j, k) < valley_find && k > valley(j, 1, h) &&
                                    imgsnc(j, k - 1) > imgsnc(j, k) &&
                                    imgsnc(j, k + 1) > imgsnc(j, k)
                                    valley(j, 3, h) = k;
                                    valley(j, 4, h) = imgsnc(j, k);
                                    break;
                            end
                        end
                    end
                    if valley(j, 3, h) == 0
                        valley_find = valley_find + 10;
                    end
                end
                if valley(j, 3, h) == 0
                    subs = subs + 5;
                end
                if valley(j, 3, h) == 0
                    subs = 5;
                    for x = 1:1:10
                        if subs < ((borders(j, 2, h) - borders(j, 1, h)) / 2);
                            valley_find = -200;
                            for u = 1:1:1000
                                if valley_find < -100
                                    sinir = (borders(j, 2, h) - subs);
                                    for k = borders(j, 2, h):1:sinir
                                        if (borders(j, 2, h) + borders(j, 1, h) / 2)
                                            if imgsnc(j, k) < valley_find && k > valley(j, 1, h) &&
                                                imgsnc(j, k - 1) > imgsnc(j, k) &&
                                                imgsnc(j, k + 1) > imgsnc(j, k)
                                                valley(j, 3, h) = k;
                                                valley(j, 4, h) = imgsnc(j, k);
                                                break;
                                        end
                                    end
                                end
                            end
                        end
                    end
                end
            end
        end
    end
end
if valley(j,3,h)==0
    valleyfind=valleyfind+10;
end
end
if valley(j,3,h)==0
    subs=subs+5;
end
end
end
if valley(j,3,h)==0
    subs=5;
for x=1:1:10
    if subs<((borders(j,2,h)-borders(j,1,h))/2);
        valleyfind=-100;
        for u=1:1:1000
            if valleyfind<-40
                sinir=(borders(j,2,h)-subs);
                for k= borders(j,2,h):-1:sinir
                    if imgsnc(j,k)<valleyfind && k> valley(j,1,h) &&
                        imgsnc(j,k-1)>imgsnc(j,k) &&
                       _imgsnc(j,k+1)>imgsnc(j,k)
                        valley(j,3,h)=k;
                        valley(j,4,h)=imgsnc(j,k);
                        break;
                    end
                    end
                end
            end
        end
    end
    if valley(j,3,h)==0
        valleyfind=valleyfind+10;
    end
end
if valley(j,3,h)==0
    subs=subs+5;
end
end
end
end
delete noise(j,2,h)=valley(j,3,h);
end
% The gradient at the left side of the left minimum should be negative
% and at the right side of the second minimum (valley) positive. Ignore
% the matrix cells which does not show this trend.
for j=cellbeginframe:1:cellendframe
    if borders(j,1,h)~=0
        for k= valley(j,1,h):-3:-1: 1
            gradient_ints(j,k,h)= imgsnc(j,k+1)-imgsnc(j,k);
            if gradient_ints(j,k,h)>0
                imgsnc(j,k,h)=0;
            end
        end
    end
end
end
end
for j=cellbeginframe:1:cellendframe


if borders(j,1,h)~0or k= valley(j,3,h)+3:1:b-1
    gradient_ints2(j,k,h)= imgsqnc(j,k)-imgsqnc(j,k+1);
    if gradient_ints2(j,k,h)>0
        imgsqnc(j,k+1)=0;
    end
end
end

% Find the second "temporary" right and left biological cell borders after noise reduction.
for j=cellbeginframe:1:cellendframe
    if borders(j,1,h)~0 & (valley(j,1,h)~0 & valley(j,3,h)~0)
        cellin=0;
        for k=1:1:b-1
            if imgsqnc(j,k)==0 & imgsqnc(j,k+1)~=0
                count=1;
                for t=k+1:1:b
                    if (imgsqnc(j,t)~=0)
                        count=count+1;
                    end
                    if imgsqnc(j,t)<-125
                        cellin=1;
                    end
                    if (imgsqnc(j,t)==0) & count<12 & cellin==0
                        count=1;
                        cellin=0;
                        break;
                    end
                    if (imgsqnc(j,t)==0) & count<12
                        count=1;
                        cellin=0;
                        break;
                    end
                    if (imgsqnc(j,t)==0) & count>12 & cellin==0
                        count=1;
                        cellin=0;
                        break;
                    end
                    if imgsqnc(j,t)==0 & count>12 & cellin==1;
                        endborders(j,1,h)=k;%which pixel is beginborder
                        endborders(j,2,h)=t;%which pixel is endborder
                        count=1;
                        cellin=0;
                        break;
                    end
                end
            end
        end
    end
end
% A threshold is given here. The matrix cells with a value higher than 
% 85% of the minima value are ignored. This is a threshold chosen 
according the experiments. The reason of this procedure is as follows: 
the matrix cells with a minimum negative value correspond to the 
biological cell borders when there is no deformation. When there is a 
deformation, due to the eliptic shape and the noise the minima may 
shift in some cases into the direction of the cell interior. In such 
% cases the matrix cells with a value of 85% of the minima are taken as 
% the biological cell borders, if they exist after noise reductions.

[sct clt]=size(imgsqnc);
for sc=cellbeginframe:1:cellendframe
  if endborders(sc,1,h)==0
    for cl=deletenoise(sc,1,h):-1:1
      if imgsqnc(sc,cl)>(valley(sc,2,h)*0.85)
        imgsqnc(sc,cl)=0;
      end
    end
  end
  for cl=deletenoise(sc,2,h):1:clt
    if imgsqnc(sc,cl)>(valley(sc,4,h)*0.85)
      imgsqnc(sc,cl)=0;
    end
  end
end

% Find the real right and left biological cell borders after noise 
reduction.
for j=cellbeginframe:1:cellendframe
  if endborders(j,1,h)==0
    cellin=0;
    for k=1:1:b-1
      if imgsqnc(j,k)==0 && imgsqnc(j,k+1)==0
        count=1;
        for t=k+1:1:b
          if (imgsqnc(j,t)==0)
            count=count+1;
          end
        end
        if imgsqnc(j,t)<-125
          cellin=1;
        end
      end
      if (imgsqnc(j,t)==0) && count<12 && cellin==0
        count=1;
bhcellin=0;
bhbreak;
      end
      if (imgsqnc(j,t)==0) && count<12
        count=1;
bhcellin=0;
bhbreak;
      end
      if (imgsqnc(j,t)==0) && count>12 && cellin==0
        count=1;
      end
  end
end
cellin=0;
break;
end

if imgsqnc(j,t)==0 && count>12 && cellin==1;
    lastendborders(j,1,h)=k+0.5;%which pixel is beginborder
    lastendborders(j,2,h)=t-0.5;%which pixel is endborder
    count=1;
cellin=0;
break;
end
end
end
end
end
end

% Calculation the size of the biological cell
[v y z]= size(lastendborders);
for i=1:1:v
    if lastendborders(i,1,h)== 0
        Sizeofcell(i,1,h)=abs(lastendborders(i,1,h)-lastendborders(i,2,h));
    else
        Sizeofcell(i,1,h)=NaN;
    end
end

% If the standard deviation of the size of the biological cell is large, it is questioned to the user. The user has to give the answer in pixels using the high speed camera program. The frames in which the size of the biological cell are two pixels more than the answer of user are ignored and the borders of the biological cell are again studied.
standev=nanstd(Sizeofcell(:,:,h));
if standev>1
    Sizeofcell(:,:,h)
    answer=input(['what is the size of the ', num2str(h) ,'.cell?? The standard deviation of the detected size is a lot!']);
    for i=1:1:v
        if Sizeofcell(i,1,h)<(answer-2) || Sizeofcell(i,1,h)>(answer+2)
            Sizeofcell(i,1,h)=NaN;
            valley(i,1,h)=0; valley(i,3,h)=0;
            endborders(i,1,h)=0;endborders(i,3,h)=0;
            lastendborders(i,1,h)=0;lastendborders(i,3,h)=0;
        end
    end
end
% Running the code again to find the biological cell borders
for j=cellbeginframe:1:cellendframe
    if borders(j,1,h)==0 && (valley(j,1,h)==0 && valley(j,3,h)==0)
        cellin=0;
        for k=1:1:b-1
            if imgsqnc(j,k)==0 && imgsqnc(j,k+1)~0
                count=1;
                for t=k+1:1:b
                    if (imgsqnc(j,t)~0)
                        count=count+1;
                    end
                    if count>12
                        cellin=0;
                        break;
                    end
                end
            end
        end
    end
end
if imagesqnc(j,t)<=125
cellin=1;
end

if (imagesqnc(j,t)==0) && count<12 && cellin==0
count=1;
cellin=0;
break;
end

if (imagesqnc(j,t)==0) && count<12
count=1;
cellin=0;
break;
end

if (imagesqnc(j,t)==0) && count>12 && cellin==0
count=1;
cellin=0;
break;
end

if imagesqnc(j,t)==0 && count>12 && cellin==1;
endborders(j,1,h)=k; %which pixel is beginborder
endborders(j,2,h)=t; %which pixel is endborder
count=1;
cellin=0;
break;
end
end
end
end
end
end
end
for sc=cellbeginframe:1:cellendframe
if endborders(sc,1,h)==0
for cl=deletenoise(sc,1,h):-1:1
if imagesqnc(sc,cl)>= (valley(sc,2,h)*0.85)
imagesqnc(sc,cl)=0;
end
end
for cl=deletenoise(sc,2,h):1:clt
if imagesqnc(sc,cl)>= (valley(sc,4,h)*0.85)
imagesqnc(sc,cl)=0;
end
end
end
end
end
end
for j=cellbeginframe:1:cellendframe
if endborders(j,1,h)==0
cellin=0;
for k=1:1:b-1
  if imgsqnc(j,k)==0 && imgsqnc(j,k+1)~=0
    count=1;
    for t=k+1:1:b
      if (imgsqnc(j,t)~=0)
        count=count+1;
      end
      if imgsqnc(j,t)<-125
        cellin=1;
      end
    end
    if imgsqnc(j,t)==0 && count<12 && cellin==0
      count=1;
      cellin=0;
      break;
    end
    if (imgsqnc(j,t)==0) && count<12
      count=1;
      cellin=0;
      break;
    end
  end
  if (imgsqnc(j,t)==0) && count>12 && cellin==0
    count=1;
    cellin=0;
    break;
  end
  if imgsqnc(j,t)==0 && count>12 && cellin==1;
    lastendborders(j,1,h)=k+0.5;  
    %which pixel is beginborder
    lastendborders(j,2,h)=t-0.5;  
    %which pixel is endborder
    count=1;
    cellin=0;
    break;
  end
end
end
end
end
end
% The middle point of the biological cell borders is calculated.
% It is used to calculate the biological cell velocity.
[v y z]= size(lastendborders);
for i=1:1:v
  if lastendborders(i,1,h)== 0
    Middlepoint(i,1,h)=(lastendborders(i,1,h)+lastendborders(i,2,h))/2;
    Sizeofcell(i,1,h)=abs(lastendborders(i,1,h)-
    lastendborders(i,2,h));
  else
    Middlepoint(i,1,h)=NaN;
  end
Sizeofcell(i,1,h)=NaN;
end
end
% The biological cell velocity is calculated.
for i=2:1:v
    if Middlepoint(i,1,h)~= 0
        Velocityofcell(i,1,h)= Middlepoint(i,1,h)-Middlepoint(i-1,1,h);
    else
        Velocityofcell(i,1,h)=NaN;
    end
end
Velocityofcell(1,1,h)=NaN;
AverageCelldata(1,1,h)= nanmean(Sizeofcell(:,1,h)) * pixsize; % cell size in micron
AverageCelldata(1,2,h)= nanmean(Velocityofcell(:,1,h))/0.0005*pixsize; % cell velocity in um per sec
end
% Display the biological cell border positions in all frames as a matrix.
figure;
[a b]=size(imgsqnc);
imagesc(imgsqnc(1:a,1:b)), figure(gcf)
colormap(gray); hold on
% Plot a middle point between the biological cell borders.
for j=1:1:z
    for i=1:1:v
        if Middlepoint(i,1,j)~= 0
            plot(Middlepoint(i,1,j),i,'.', 'LineWidth',1,'MarkerEdgeColor','r','MarkerSize',8)
        end
    end
end
end