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Biological Cell Models and Atomic Force Microscopy: A Literature Review

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Master of Science in Cybernetics and Robotics
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MSc thesis assignment

Name of the candidate: Kine Iversen

Subject: Engineering Cybernetics

Title: Biological Cell Models and Atomic Force Microscopy: A Literature Review

Background

Atomic force microscopy (AFM) is one of the foremost tools for imaging, measuring and manipulation at the nanometer scale. The AFM can be utilized for studying different kinds of materials, also biological cells. In this study the aim is to review the scientific literature on mechanical models of cells, with focus on unknown parameters in such models.

Assignment:

1. Present the working principles of AFM
2. Perform a literature review on mechanistic models of biological cells. Of particular interest are models that are suited for parameter identification. Summarize the findings and compare the different models.

To be handed in by: 21/12-2015
Cosupervisor(s): PhD-student Michael Ragazzon, ITK

Trondheim, 4/8-2015

Jan Tommy Gravdahl
Professor, supervisor

Starting point for literature:

- Dropbox-folder of papers collected by M. Vagia

Also relevant:

- PhD-thesis of A. Eielsen
- MSc-thesis of M. Ragazzon
- MSc-thesis of J.Å. Stakvik

Preface

This thesis is the final work done to complete my master's degree in Engineering Cybernetics at NTNU, carried out during the autumn semester of 2015. After five (and a half) years of hard effort, it will be a delight to enter a new chapter in my life. I will bring along excellent academic knowledge, but also lessons learned about myself.

First of all, I would like to thank my supervisor Professor Jan Tommy Gravdahl for his guidance, flexibility, and backing throughout this work. Also, my co-supervisor, PhD-student Michael Ragazzon has contributed with great prior knowledge and I would like to thank him for his presence to answer my questions at all hours.

In addition, I would like to thank my fellow students, a remarkable group of people with close ties. A special thanks go to my parents for all the past, present and future support.

Kine Iversen
Trondheim, December 2015

Abstract

Mechanical properties of cells can be used in diagnostics of various diseases. It has been proven, by several independent research groups, that sick and healthy cells differ in stiffness. Being able to extract this information will help bring medicine forward.

The Institute of Engineering Cybernetics at NTNU is in possession of an Atomic Force Microscope, which can be used to image and probe cells. They aspire to use their knowledge within parameter identification to identify and estimate unknown parameters in models of biological cells. The first step in this process is to obtain an overview of existing models and see which of them that are suited for this purpose. This has been the aim of this thesis.

The work conducted on cell mechanics either views the cell as an elastic material or a viscoelastic material. Due to these different interpretations, the field appears confusing. The cell is viscoelastic, but it can be approximated as elastic to simplify calculations. In this work, both these modelling approaches are discussed. Comments about parameter estimation have also been included to make this thesis an adequate basis for further work on this topic.

The target group of the thesis are readers with a mathematical understanding, but limited knowledge about biology and cell mechanics. However, this review can be useful for anybody that is interested as no existing work is comprehensive enough in the discussion of both elastic and viscoelastic models. By the end of the thesis, the reader will have obtained an overview of the field concerning cell mechanics. If a deeper insight is desired, the bibliography and a list of the most important articles found in the Appendix can serve as an excellent utility.

For the cell to yield a linear response, Atomic Force Microscopy experiments need to apply sufficiently small forces. Though this is a simplification of the reality, the corresponding models will be easier to perform parameter estimation on. They serve as a good starting point in the further work on parameter estimation in mechanical models of cells.

Sammendrag

De mekaniske egenskapene til celler kan brukes i diagnose av forskjellige sykdommer. Flere uavhengige forskningsgrupper har bevist at syke og friske celler har ulik stivhet. Å ha muligheten til å hente ut denne informasjonen fra celler vil bidra til å føre medisin fremover.

Institutt for teknisk kybernetikk ved NTNU er i besittelse av et Atomic Force Microscope, et mikroskop som kan brukes til å avbilde og undersøke celler. De ønsker å benytte sine kunnskaper innen parameteridentifisering til å identifisere og estimere ukjente parametre i modeller for biologiske celler. Det første steget i denne prosessen er å få en oversikt over allerede eksisterende modeller og se hvilke av dem som egner seg til denne bruken. Dette har vært formålet med denne masteroppgaven.

Arbeidet utført på cellemekanikk ser enten på cellen som et elastisk materiale eller et viskoelastisk materiale. På grunn av disse ulike tolkningene fremstår feltet forvirrende. Cellen er viskoelastisk, men den kan modelleres som elastisk for å forenkle beregninger. I dette arbeidet er begge disse tilnærmingene vurdert. Kommentarer om parameterestimering er også tatt med for å gjøre denne oppgaven til et tilstrekkelig utgangspunkt for videre arbeid på dette feltet.

Målgruppen for oppgaven er lesere som innehar matematisk forståelse, men med begrenset kunnskap om biologi og cellemekanikk. Likevel kan denne gjennomgangen være nyttig for alle som er interessert, ettersom det ikke finnes eksisterende arbeid som er omfattende nok i diskusjonen av både elastiske og viskoelastiske modeller. Etter å ha lest denne oppgaven vil leseren ha fått en oversikt over feltet som omhandler cellemekanikk. Hvis en dypere innsikt er ønskelig, kan litteraturlisten og vedlegget som oppsummerer de viktigste artiklene være gode verktøy.

Hvis cellen skal ha lineær respons må eksperimentene med Atomic Force Microscope anvende tilstrekkelig små krefter. Selv om dette er en forenkling av virkeligheten, vil de tilsvarende modellene være lettere å utføre parameterestimering på. De fungerer som et godt utgangspunkt i det videre arbeidet med å gjøre parameterestimering i mekaniske cellemodeller.

Table of Contents

List of Figures

Nomenclature

b	drag factor
j_0	compliance at $t = 0$
l_o	original length
Δl	extended length relative to l_o
v	Poisson's ratio
A	cross-sectional area
E	Young's modulus
E_{rel}	relaxation modulus
F	load force
G	shear modulus
G^*	complex shear modulus
$G^{'}$	storage modulus
$G^{''}$	loss modulus
G_0	scaling factor for stiffness
J	creep compliance
R	radius of the intender
α	power-law exponent in power-law structural damping model
β	power-law exponent in power-law rheology

ε	normal strain
ε_0	normal strain at $t = 0$
σ	normal stress
σ_0	normal stress at $t = 0$
θ	opening angle for a conical tip
γ	shear strain
ϕ	shear stress
τ	relaxation time
μ	viscosity
η	damping coefficient in power-law structural damping model
δ_s	indentation depth
δ_c	cantilever deflection
δ_0	operating indentation during oscillations
φ	phase lag
ω	radian frequency of oscillation
ω_0	scaling factor for frequency
Γ	Gamma function
AFM	Atomic Force Microscopy
CP	contact point

Chapter 1

Introduction

1.1 Background

The field of cybernetics is the science of dynamical systems' behaviour and how to control and monitor them autonomously. This includes robots, ships, engines, industrial processes and many more. A particular strength is the ability to describe systems with mathematical models and then do parameter estimation on unknown quantities and perform simulations to see how the system behaves. The field is provident and has the capability to contribute in other disciplines by merging knowledge. One example of this is Atomic Force Microscopy (AFM) used in biology and medicine to study cell mechanics.

AFM is a microscopy technique used to image and manipulate cells. It can also reveal their mechanical properties. This is interesting knowledge. It has for instance been proven that healthy and diseased cells differ in stiffness (Lim et al., 2006; Guz et al., 2014; Haase and Pelling, 2015). To obtain this information, it is necessary to have access to mechanical models of the cell. A lot of work and effort have been conducted on this by numerous researchers, and there is to a certain extent agreement on models that can explain how cells respond to externally applied forces.

Previous work from cyberneticians on AFM has been related to control on the nanoscale, which has lead to improved scanning speed during experiments. Now, there is a desire to look into mathematical modelling of cells to potentially contribute to identify and estimate unknown parameters. This is not a well-developed research area in AFM context, where most of the techniques used are curve fitting and statistical analysis. Engineering Cybernetics can contribute with knowledge about dynamics, robotics and parameter identification to help bring this discipline forwards. To be able to do this, it is important to examine existing mechanical models of cells, which is knowledge not held by the average cybernetic researcher. The aim of this thesis is to present an overview of the leading theories on mechanical modelling of cells that can be used in the analysis of data collected

from AFM experiments. The contribution will be an extensive bibliography for this field of study and a listing of the most relevant articles.

1.2 Limitations

This thesis will look at models that only are valid when cells are applied to small forces, which results in a linear response. Even if the model itself has a nonlinear form, all models discussed here will assume this. Consequently, there will be no evaluation of models that describe cell's nonlinear behaviour. This assessment is done because there are limited existing descriptions for this kind of models. Also, they are too complex for a gentle start considering parameter estimation.

When studying the mechanical response of an entire cell population, rare or transient phenomena can be obscured when one averages together the response of individual cells (Rodriguez et al., 2013). Because of this, the research will be limited to models of single cells, and the mathematical description of cell populations are omitted. This also fits better with AFM, which can only probe one cell at the time.

1.3 Approach

When performing a literature review, there are a lot of articles to be read and considered. In the start of the work, critical analysis is not possible as many mechanical and biological terms need to be understood and defined. After gaining a better grasp of this, it will be essential to excrete the articles that are not relevant and focus on the articles that discuss mechanical models that are possible to use together with AFM. The most promising methods and results will be further investigated by viewing the bibliography of the articles that examine them, but also take a look at work that have cited these particular articles.

The articles with a higher number of citations will be considered significant contributions to the field. However, there should be a desire to obtain an overview that is up-to-date. This will be achieved by looking at what kind of research that has been conducted in this area the recent years. In these cases, the focus on multiple citations must be discarded as there has not been enough time for the articles to attain this.

Search engines are powerful utilities in a literature review. Google Scholar will be a helpful tool to see quickly how many citations an article has. Databases like Web of Science, Scopus and Oria, where the latter is NTNU's library overview, are a better fit when searching with proximity operators, truncations and Boolean operators to limit the search. A combination of these approaches will by used in this work.

A goal in this literature review will be to, as far as possible, mainly base the content on peer-reviewed articles.

Citations will be presented in Harvard style, as this will make the text easier to read. This way, it is possible for the reader to see patterns of repeating articles.

Because some of the material in the bibliography is not directly relevant for cell mechanics and AFM as they are being used for other definitions, there will be a table in the Appendix that presents an overview of the most prominent ones. Together with the bibliography, this thesis will be a great starting point for scientists that are interested in an overview of the field concerning cell mechanics and AFM.

1.4 Outline

In chapter 2, the relevant theory about cells and definitions in mechanics is presented. These concepts and terms may be familiar to biologists, but necessary for non-experts in the field.

Next, in chapter 3, follows an extensive description of AFM and its working principles.

A review of cell mechanics in general can be found in chapter 4. Chapter 5 and chapter 6 then elaborates on elasticity and viscoelasticity calculations respectively.

The current challenges within the description of cell mechanics are the content of chapter 7, while chapter 8 and chapter 9 contains summary and conclusions for this thesis.

Chapter 2

Theory

2.1 Biological Cells

Cells are the foundation of all living tissue and organs. The cell is alive itself and is thus the smallest of all living substances in humans and animals. They have all the fundamental features of an organic life: metabolism and the ability to move and reproduce.

There is a distinction between prokaryotic and eukaryotic cells. "The simplest of simple organisms" is a description of the prokaryotic cell, most of them sized around 1μm. They are mainly bacteria and are not, in this context, as interesting as the larger eukaryotic cells ($10 - 100\mu$m), found in plants and animals (Cooper and Hausman, 2009). A eukaryotic cell has several components called organelles (little organs). Also, it contains structures called cytosol and cytoplasm. A description of essential constituents of a cell follows, where much of the information is gathered from (Rodriguez et al., 2013).

- **Nucleus**
  The nucleus if often referred to as the cell's brain. It can occupy up to 10 percent of the space inside a cell and contains DNA which determines the cell's identity and masterminds its activities.
- **Cytosol**
  The cytosol of a cell is its interior, excluding the organelles. It is a semi-fluid solution of proteins, salts and other molecules.
- **Cell membrane**
  The barrier between the cytosol and the extracellular environment. Biological membranes also enclose organelles and control the passage of materials into and out of them.
- **Cytoplasm**

The material between the membrane and the nucleus. It includes all the organelles and the cytosol, except the nucleus. The previously described cytosol is the fluid portion of the cytoplasm.

- **Cytoskeleton**
  With the objective to study cell mechanics, the cytoskeleton is an essential part of the cell because it yields shape and support. The cytoskeleton lies within the cytoplasm and consists of different filamentous proteins: microtubules, intermediate filaments and actin filaments. Out of these three, the actin filaments are central. They are integrated into the cytoskeleton designed principally to reinforce the cell against mechanical deformation and to allow for force generation, leaving them as key components of the mechanical support of eukaryotic cells.

The study of cells can happen *in vitro*, *in vivo* or *in silico*. *In vitro* studies happen outside the living organism, often in a laboratory, while *in vivo* happens within the biological context. The advantages of doing experiments *in vitro* are that it is faster, less expensive and that scientists can conduct studies on specific cells instead of the organism as a whole. The downside is that the results do not necessarily translate well to real life. Another type of approach is *in silico* biology, which refers to the use of computers to perform biological studies. As pointed out by (Palsson, 2000), many other fields of science and engineering have developed systems science and complicated mathematical simulations to a high level of sophistication, but biology is lagging behind.

2.2 Mechanical Expressions

2.2.1 Stress and strain

To know how the forces acting on a body will deform it is an important mechanical property of a material. There are two key terms here; stress and strain.

Stress is the applied force on a body and is defined as either compressive, tensile or shear stress, see figure 2.1. Compressive stress pushes the body with forces while the tensile stress stretches it. In both cases, the forces are perpendicular to the area they act on, and they are referred to as normal stress. It has the definition:

$$\text{normal stress} = \sigma = \frac{F}{A} \tag{2.1}$$

where F is the applied force and A is the cross-sectional area of the material, giving stress the unit $[N/m^2]$ or $[Pa]$.

Shear stress, on the other hand, works differently on a body because the applied forces are parallel to the plane, and they do not share the same line of action.

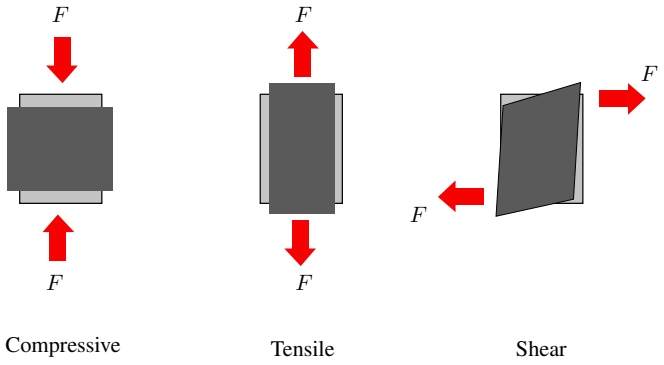

Figure 2.1: The various types of stress

$$\text{shear stress} = \phi = \frac{F}{A} \tag{2.2}$$

The other important term in this section is strain, which is a quantification of the stress applied. It indicates how much extension there is per unit length and is given by the formula

$$\text{normal strain} = \varepsilon = \frac{\Delta l}{l_o} \tag{2.3}$$

where Δl is the extended or decreased length of the material and l_o is the original length. This is a unitless property. As for shear strain, the formula is equal, but the length difference is the movement of the cross-sectional area as a response to the applied forces. Δx is used instead of Δl to avoid misunderstandings.

$$\text{shear strain} = \gamma = \frac{\Delta x}{l_o} \tag{2.4}$$

2.2.2 Rigidity, elasticity, plasticity and viscosity

Rigidity is the relative stiffness of a material that allows it to resist bending, stretching, twisting or other deformation under a load (BusinessDictionary, n.d.).

Elasticity is a non-permanent deformation where the material recovers to its original shape when the applied stress is removed. The elastic response of the cell is mainly due to its cytoskeleton (Radmacher, 1997).

Plasticity is a property of a material that allows it to deform irreversibly. It has some similarities to elasticity, but without the ”recovery” when removing the load. A body made

out of plastic material can thus change its shape easily by the application of appropriately directed forces, and retain the new shape upon removal of such forces (Lubliner, 1990).

Viscosity is the quantity that describes a fluid's resistance to flow (Elert, n.d.). A fluid with large viscosity, like e.g. honey, will resist motion better than a fluid with lower viscosity, like water. Viscosity is denoted μ in this thesis, but note that many articles use η.

Materials composed of both rigid-like (elastic) and fluid-like (viscous) elements are characterized as *viscoelastic* (Cameron et al., 2014). A viscoelastic material will return to its original shape after the load is removed, i.e. it will show an elastic response. However, it may take time to do so because of the viscous component (Vincent, 2012). Cells belong to this category of materials as they possess both behaviours (Kollmannsberger and Fabry, 2011). If cells were purely elastic they would not be able to perform operations like spreading and division and a purely viscous cell would be unable to maintain its structural integrity (Fabry et al., 2001).

2.2.3 Young's modulus and shear modulus

An important aspect of stress and strain is the relationship between them. The ratio between tensile stress and strain of a material is constant for a particular range of loads, see figure 2.2.

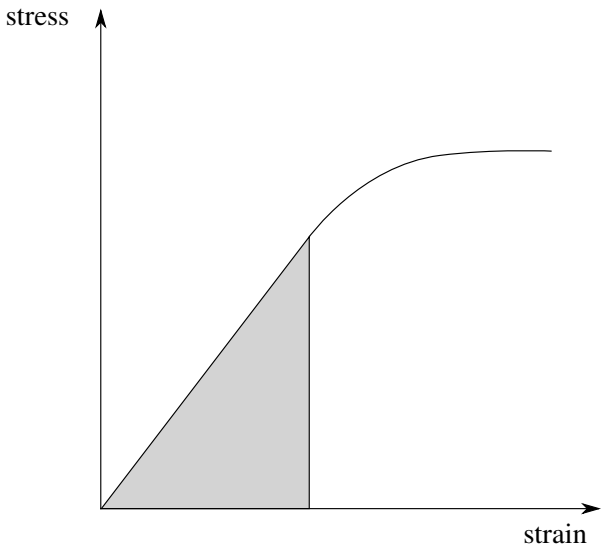

Figure 2.2: Example of a stress-strain curve

This linear portion is called Young's modulus, or modulus of elasticity, and measures the resistance of the material against elastic deformation. It is denoted E and given by the gradient of the correlation between stress and strain:

$$E = \frac{\text{tensile stress}}{\text{tensile strain}} = \frac{\sigma}{\varepsilon} = \frac{Fl_o}{A\Delta l} \tag{2.5}$$

Note that as long as the stress-strain relation is linear, the deformation of the material is elastic. However, when the curve in figure 2.2 deviates from linearity, the material enters the plastic deformation stage that causes permanent changes in shape from the applied stress (Vinckier and Semenza, 1998).

Larger gradient and Young's modulus equal a stiffer material. For most substances this quantity is known, but not for cells because they are more complex and have varying stiffness. Hard materials like glass and steel can have a Young's modulus of ≈ 100 GPa while cells are somewhere between 1 kPa and 100 kPa (Radmacher, 1997). In chapter 5 there will be a description of how to calculate this number.

The ratio between shear stress and strain is called the shear modulus or modulus of rigidity and is given by

$$G = \frac{\text{shear stress}}{\text{shear strain}} = \frac{\phi}{\gamma} = \frac{Fl_0}{A\Delta x} \tag{2.6}$$

The shear modulus of solids is independent of frequency while that of liquids is proportional to frequency. Because cells display viscoelastic behavior, there exists a frequency-dependent variation of the shear modulus called dynamic shear modulus, $G^*(\omega)$. This is an indicator of overall viscoelastic behaviour (Moeendarbary and Harris, 2014). It is also referred to as the complex shear modulus when expressed as a complex quantity and calculated doing oscillatory measurements over a wide frequency range. The frequency-dependent shear modulus will be further explained and discussed in section 6.4 when looking at viscoelastic power-law models.

There also exist a relationship between Young's modulus and the shear modulus, valid for linear elastic materials (Lim et al., 2006).

$$E = 2(1 + v)G \tag{2.7}$$

with v being the Poisson's ratio, giving a numerical value to the changes in dimensions that occur when stretching a material. Equation (2.7) is not adequate for describing the mechanics of cells, because the elasticity of a viscoelastic material will depend on both the loading rate and loading history (Lim et al., 2006), but it can be used as an approximation. Poisson's ratio is given by

$$v = -\frac{\text{lateral strain}}{\text{longitudinal strain}} \tag{2.8}$$

This value will always be between 0 and 0.5 (Vinckier and Semenza, 1998). According to (Sokolov, 2007; JPKinstruments, n.d.) the majority of biological material will have the Poisson ratio $v = 0.5$.

2.2.4 Stress relaxation and creep

A large amount of the information in this section is from (Roylance, 2001).

While stress and strain can describe elastic materials, the mathematical description of viscoelastic materials involves the introduction of a new variable - time. To model the behaviour of materials with both viscous and elastic components, one can use stress relaxation and creep experiments. Both these are transient procedures. As defined in (Lopez-Guerra and Solares, 2014) and shown in figure 2.3 and 2.4, stress relaxation is the time-dependent drop in stress under constant strain, while creep is the time-dependent strain relaxation under a constant stress.

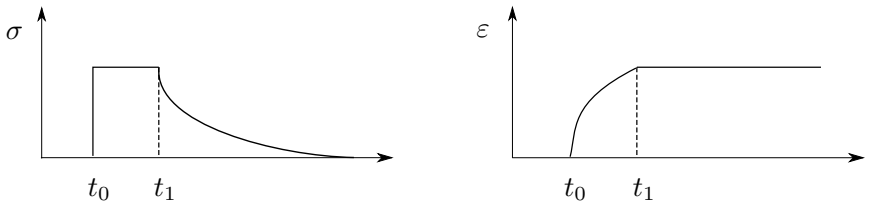

Figure 2.3: We can see stress relaxation from t_1 where the strain is kept constant.

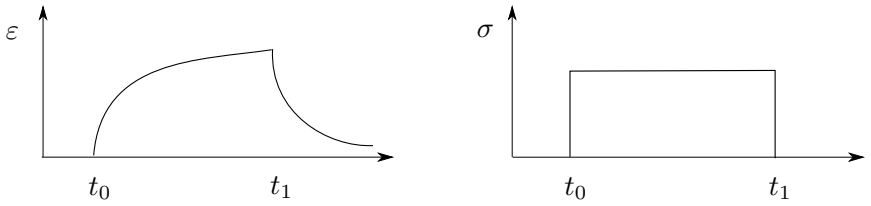

Figure 2.4: Creep curve with recovery. A constant load is applied at t_0 and removed at t_1. Notice that in this example the strain does not recover completely to its initial value, which means that the deformation is permanent and that the material is plastic.

The alternative to transient experiments is dynamic procedures, where stress or strain is varied cyclically with time. Then, the response is measured at various frequencies of deformation (Vincent, 2012). In this section, however, the focus is on the transient experiments, namely stress relaxation and creep.

Creep
Creep is the change of deformation under a load and how this evolves over time. During constant stress experiments, creep compliance, J, can be measured. Compliance is the inverse of stiffness (Haase and Pelling, 2015) given as

$$J(t) = \frac{\varepsilon(t)}{\sigma_0} \tag{2.9}$$

This is used in the case where a time-varying strain, $\varepsilon(t)$, arises from a constant stress, σ_0. Often, the creep compliance is plotted against the logarithm of time. In figure 6.2 the point on the x-axis labeled "log τ" marks the inflection from rising to falling slope, and τ is the relaxation time of the creep process (Roylance, 2001).

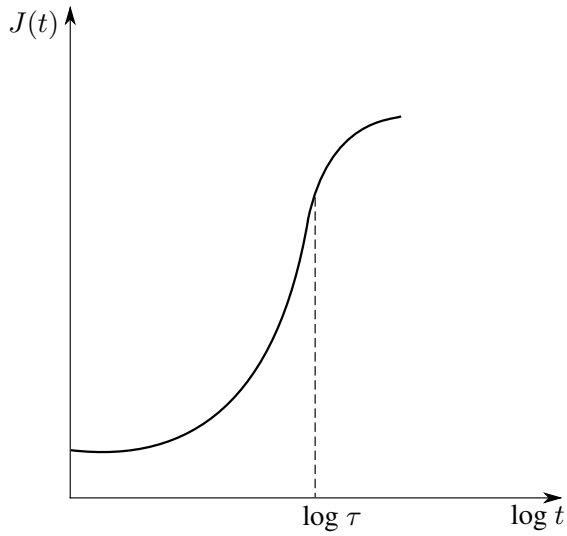

Figure 2.5: Creep compliance plotted against the logarithm of time

Stress relaxation
In the other mentioned technique, stress relaxation, the material is deformed, and the force required to maintain the deformation at a constant value is measured. The stress required dies away with time and is said to relax (Vincent, 2012). Similar to creep compliance there exists a relaxation modulus (Roylance, 2001)

$$E_{rel}(t) = \frac{\sigma(t)}{\varepsilon_0} \tag{2.10}$$

where ε_0 is the strain fixed at a constant value. The relaxation can be plotted against logarithmic time in a similar manner to creep.

2.3 Parameter Estimation

The general problem (Zhang, 1997) is that the unknown parameters are gathered in a vector **p**, whose dimension indicates the number of parameters to be estimated. The output

of the modeled system is assembled in a measurement vector **z**. A noise-free case, a simplification done here, relates **z** to **p** so that

$$\mathbf{f}(\mathbf{p}, \mathbf{z}) = 0 \tag{2.11}$$

The desire is to use the observed measurements, **y** = **z**, to estimate **p**.

One approach is to use curve fitting. By minimizing the square of the error between experimental data and established models, unknown parameters can be estimated. This method is called least squares fitting.

To ease the calculations of the unknown parameters, it is preferable that they are linearly given in the expressions.

Chapter 3

Atomic Force Microscopy

In optical microscopy, a lens is used to magnify an image, and this is what most people associate with the term microscope. Even though this can be a useful tool in a broad range of applications, it has a maximum resolution of about 100 nm (Abramovitch et al., 2007). Sometimes, especially when studying cells, it can be interesting to view objects down to atomic scale and also be able to do nanomanipulation on the sample. As shown in (Abramovitch et al., 2007), Atomic Force Microscopy (AFM) is one of the leading and most versatile methods for imaging nanoscale structures after its invention and introduction by (Binnig et al., 1986) in the 1980's. This is due to its resolution with the ability to see individual atoms and that the imaging environment is flexible. See section 3.2 for a more thorough discussion of the pros and cons of AFM.

Some scientists saw the potential of AFM to be used in biological studies already in the early 1990s. They hoped to get the opportunity to capture microscopic images of biological phenomena in vivo, but the development in the field was slow. This was partly because, at that time, AFM was only applicable to samples in air. In vivo experiments were dependent on imaging under fluid. With the further development and improvements of AFM, the widespread use of the technique within biology started around the 2000s (Takeyasu, 2014).

Other techniques being used to study cell's mechanics are, to mention some, micropipette aspiration (Hochmuth, 2000), optical tweezers (Dao et al., 2003; Zhang and Liu, 2008), magnetic beads (Haukanes and Kvam, 1993) and cytointender (Shin and Athanasiou, 1999). However, no further description will be presented here. If the reader is interested in details about how they work, the cited articles serve as excellent sources of information.

To understand the potential of AFM used in combination with mechanical models of cells, a description of the technique is necessary. This chapter explains the working principles of AFM and its modes of operation. Also, the information about cells that is possible to

extract from AFM experiments is discussed. In the last section, 3.2, AFM benefits and drawbacks are described.

3.1 Working Principles

The atomic force microscope consists of the components shown in figure 3.1. A probe with a very sharp tip is connected to a cantilever and scans over a sample surface, causing interaction forces between the tip and the surface. A laser beam is directed towards the back of the cantilever and is reflected towards a photodetector. The probe follows the contour of the sample and causes the cantilever to bend accordingly.

There are several ways of moving the tip relative to the sample. A standard approach is to use a piezo actuator to move the sample in x- y- and z-direction. The vertical movement is done in response to the deflection of the cantilever. An alternative is to do the movement in the xy-plane by maneuvering a stage beneath the sample and control the z-axis by moving the cantilever up and down (Abramovitch et al., 2007). To read more about issues related to the choice of control design, see (Schitter, 2007) and (Kwon et al., 2003).

Depending on the mode of operation (see section 3.1.1), either the deflection of the cantilever or its amplitude of oscillation is held constant using a feedback loop to the z-actuator. The surface estimate is given by the feedback loop itself, which in commercial systems comes from some function of the control signal and serves as a good representation of the surface topography (Abramovitch et al., 2007).

3.1.1 Modes of operation

There are three primary imaging modes in AFM: contact mode, tapping mode and non-contact mode. The latter two are dynamic methods while contact mode is static. Below follows a description of them with their benefits and drawbacks. We primarily base the material on (Wilson and Bullen, n.d.; Sokolov, 2007).

- ***Contact mode***
  The tip is ”dragged” across the surface of the sample. By keeping a constant cantilever deflection using a feedback system, the force between the sample and the probe remains constant and thereby obtain an image of the surface. The drawback is that it can scratch the sample and potentially destroy it due to high friction. Because of this, contact mode is seldom used on biological material. Advantages of this mode are its simplicity and that it allows fast scanning.

- ***Tapping mode***
  AFM tapping mode only touches the sample surface for very short periods of time because the cantilever oscillates during the measurements. The chosen oscillation frequency is the cantilever's resonance frequency or somewhere near it. Every time the tip touches the sample there is a change in the oscillation amplitude, where the change depends on the tip-sample distance. To avoid this, feedback is used to

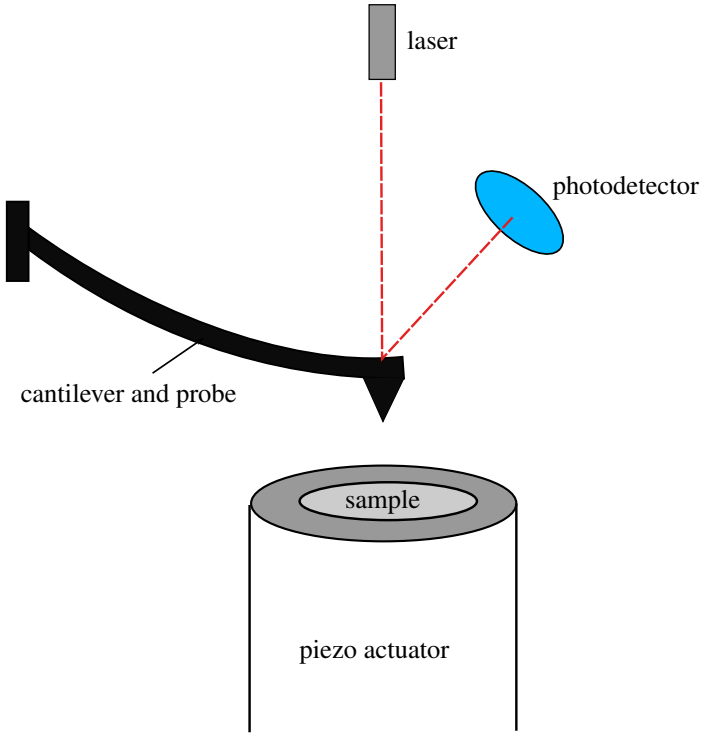

Figure 3.1: Typical AFM setup

keep the amplitude constant and thus obtain a constant tip-sample interaction. This makes it possible to create an image of the surface. This approach is typically slower regarding imaging bandwidth than contact mode because it is dependent on the slow amplitude estimate. Despite this, tapping mode is the preferred imaging method for soft biological surfaces. This is because there is lower lateral friction than in contact mode, causing less harm on the sample.

- ***Non-contact mode***
  The probe is not in touch with the sample, but oscillates above it. By using a feedback loop to monitor the changes in the amplitude due to attractive forces, the topography can be measured. The advantage of this mode is that there is less wear on the probe tip and the sample compared to the two other modes. However, non-contact mode provides the best results when operated under vacuum, which compromises its use in biology (Takeyasu, 2014).

3.1.2 Force-distance and force-indentation curves

In addition to imaging, AFM can be used in force measurement, often referred to as force spectroscopy (Takeyasu, 2014). This makes it possible to extract useful information about

the sample beyond its topography. In a force-distance curve analysis, the probe is repeatedly brought towards the surface and then retracted without scanning in the x- and y-direction. The result is a plot of the tip-sample interaction forces vs. tip-sample distance. In a stationary setting, the tip-sample interaction force is given by Hooke's law:

$$F_c = -k_c \delta_c \tag{3.1}$$

where k_c is the cantilever spring constant, calibrated and measured previous to scanning, and δ_c is the measured cantilever deflection from rest position (Capella and Dietler, 1999).

The cantilever deflection is measured with AFM. Also, the distance between the cantilever rest position and the sample, Z, can be detected because this is determined by the piezo actuator. With Z and F_c known it is possible to draw a force-distance curve, see figure 3.2 for an example. Force-distance curves consist of two parts: an approaching curve and a retracting curve. As the probe approaches the surface, the cantilever may bend upwards due to repulsive forces and the approach curve can thus be used to measure surface forces (Dufrene, 2002). See (Capella and Dietler, 1999; Heinz and Hoh, 1999) for exhaustive in-depth analysis of force-distance curves.

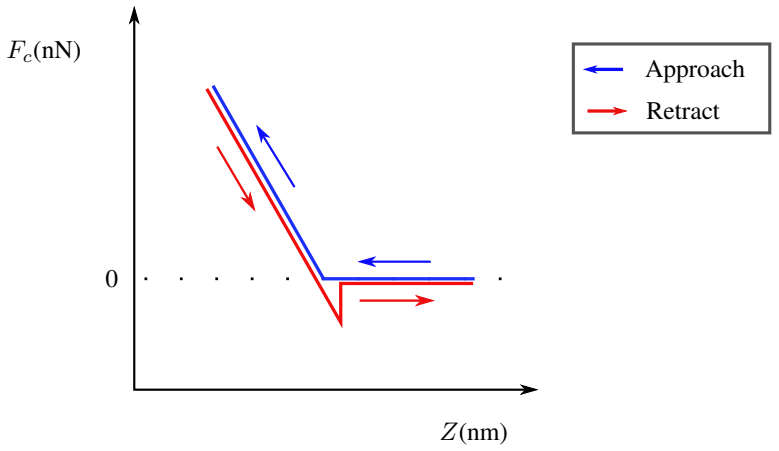

Figure 3.2: Example of force-distance curve.

From the force-distance curves, it is possible to get force-indentation curves. As (Takeyasu, 2014) explains it:

> *"In a force-distance curve, the x-axis is the measured distance Z (a measure of the piezo height), which is usually corrected to be the position of the undeflected cantilever. In a force-indentation curve, this measurement must be corrected for by taking the deflection of the cantilever into account."*

This means that the actual tip-sample distance is

$$D = Z - (\delta_c + \delta_s) \tag{3.2}$$

where δ_s is the indentation of the sample (Capella and Dietler, 1999), see figure 3.3.

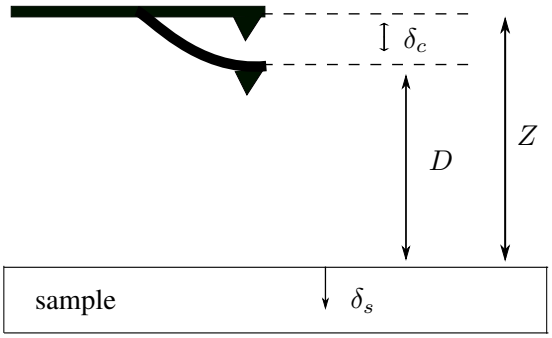

Figure 3.3: Scheme of the relevant distances in AFM. D is the tip-sample distance, Z is the distance between sample and cantilever rest position, and δ_c and δ_s are the cantilever deflection and sample indentation respectively. A copied version of Fig. 1 in (Capella and Dietler, 1999).

When $D = 0$, a force-indentation curve can be obtained (Heinz and Hoh, 1999). From these curves, it is possible to extract mechanical properties. An example is to obtain Young's modulus by fitting the curve to an elasticity model like Hertz or Sneddon (Moreno-Flores et al., 2010) as illustrated in figure 3.4. See details about this in chapter 5.

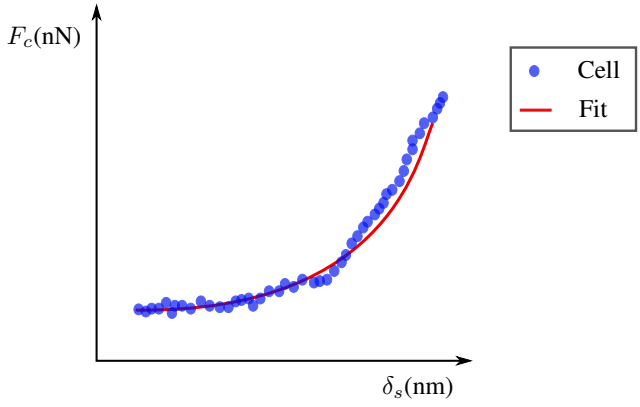

Figure 3.4: Example of a force-indentation curve from measurements (blue dots) fitted to an elasticity model (red line). Inspired by a figure from (Ramos et al., 2014).

3.2 Benefits and Drawbacks

As previously mentioned, the atomic force microscope can work in several imaging environments like air, vacuum and liquid. This is in contrast to other atomic scale microscopes that relies on vacuum to function (Abramovitch et al., 2007). Other advantages of AFM in the study of biological objects is that it can scan surfaces with up to nanometer resolution, it can provide true 3D surface topographical information and minimum preparation of the sample is required (Sokolov, 2007). However, the most important feature with our objective is that AFM allows measuring of various biophysical properties of materials.

AFM can also be used in nanomanipulation. The tip is able to apply a variety of forces, including contact, magnetic, thermal, and electrical using modified tips (Abramovitch et al., 2007).

Note that not all of these benefits are unique to AFM compared to other techniques.

Slow scanning speed is one of the main drawbacks of AFM (Abramovitch et al., 2007). Especially when collecting data to draw force-distance curves, image acquisition time can exceed 20 minutes (Pelling et al., 2007). According to (Cartagena-Rivera et al., 2015) this is 1-2 orders of magnitude longer than that required to study dynamic cellular processes.

Other challenges relate to uncertainty in the estimation of tip radius and spring constant, compression of the sample against its substrate, nonlinear loading and non-ideal sample morphology (Kurland et al., 2012). Another drawback mentioned in (Abramovitch et al., 2007) is that each measurement, each sample and each new cantilever/tip combination requires the system to be adjusted again. This makes it hard to repeat experiments because the results can vary from scan to scan (Ragazzon, 2013). Also, each operating mode has different pros and cons as explained in section 3.1.1.

Despite the drawbacks of AFM, the benefits are more substantial. As (Sokolov, 2007) puts it: *"there are few other probe methods used to study cell mechanics. The most popular ones are optical tweezers, magnetic beads and micropipette. However, those methods cannot compete with the precision that can be attained with AFM method."*

Chapter 4

Cell Mechanics in General

Cells are exposed to a variety of mechanical loads *in vivo*, both external and internal (Haase and Pelling, 2015). They are regularly on the move through dense tissue, and it is important that they can adapt their shape, but also be able to produce forces to withstand the stresses from other cells (Brunner et al., 2006). In addition to this, cells are often subjected to mechanical loads and many chemicals are known to increase or decrease the mechanical properties of living cells. Due to all these environmental and internal impacts, it is of great importance to know how the cells will respond (Lim et al., 2006).

There exist two distinct approaches to studying cellular systems, called top-down and bottom-up. The bottom-up method gathers details about different constituents of the system and builds a model based on their connections. This is hard to do with cells because their structure is complex. In the top-down approach, the starting point is a description of the entire system and then to break it down into smaller segments. In AFM context the top-down approach is more applicable (Moeendarbary and Harris, 2014) and is what the focus will be when choosing mechanical models. See (Kollmannsberger and Fabry, 2009) for a list of models developed from the bottom-up perspective.

Another distinction of models is those derived from either the micro/nanostructural approach or the continuum approach. From (Lim et al., 2006):

> *"The former deems the cytoskeleton as the main structural component. [..] On the other hand, the continuum approach treats the cell as comprising materials with certain continuum material properties. From experimental observations, the appropriate constitutive material models and the associated parameters are then derived. [..] the approach is easier and more straightforward to use in computing the mechanical properties of the cells if the biomechanical response at the cell level is all that is needed."*

In the existing literature, there are two main areas of focus within cell mechanics: measuring the elasticity of cells or measuring their viscoelasticity. The former is important

because there are several reports on a correlation between stiffness of cells and diseases like cancer, malaria, arthritis and aging (Guz et al., 2014). Young's modulus of a cell can be extracted from AFM experiments using force-indentation curves. However, as previously mentioned, cells are viscoelastic, and a description of elasticity is not adequate in describing their mechanical properties. Because of this, the viscoelastic models are more accurate, though the elastic models are satisfactory for their use. In the next chapters, which are divided into elasticity and viscoelasticity calculations, we describe the most common models available today. In addition, we will mention models without extensive details. This is either because they are used by other techniques than AFM or that they are derived from the micro/nanostructural approach.

See figure 4.1 for a visualization of the above information and which models we are discussing in this thesis. Note that there also exist, in addition to elastic and viscoelastic models, a biphasic model. This model will only be briefly described in section 6.6.3 since it is not widely used.

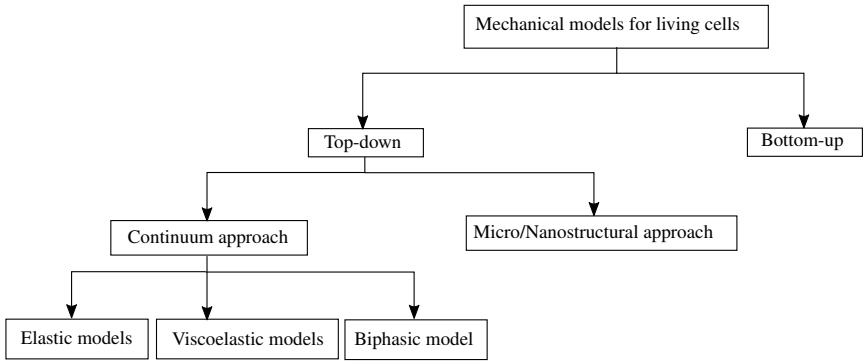

Figure 4.1: The models we have chosen to look at in this thesis, which is top-down models derived with the continuum approach. The chart is an extended version of figure 1 in (Lim et al., 2006).

Chapter 5

Elasticity Calculations

It has been shown that cells can change their elasticity quite considerably due to different diseases, and it has been increasingly common to identify and characterize sick and healthy cells using stiffness measurements (Haase and Pelling, 2015). It is possible to determine the elasticity of cells by looking at the force applied by the AFM tip and the resulting deformation (Vinckier and Semenza, 1998). Collecting the force curves over a particular area provides the ability to create an elasticity map of the cell surface (Kuznetsova et al., 2007).

The elasticity models that will be discussed in the next sections can be used in combination with force-indentation curves made from an AFM scanning. Software, e.g. Matlab, fits the mechanical models to experimental data. Young's modulus, E, can then be derived using these equations by different methods. Usually, it is calculated by fitting to the force-indentation curves using E as a fit parameter. The contact point and baseline can also be used as variable fit parameters, or they can be determined beforehand and used as fixed values (JPKinstruments, n.d.).

5.1 The Hertz Model

A favoured mechanical model used to measure the elasticity of cells with AFM is the Hertz model, based on Hertz theory of elastic contact (Benitez and Toca-Herrera, 2014). The Hertz model is only valid when the indentation depth is no more than $\sim 10\%$ of the sample thickness and when the indentation depth is > 200nm (Pelling et al., 2007). The result of this is that measurements are restricted to the central region of the cell (Gavara and Chadwick, 2012). The model also assumes that the material is isotropic, homogeneous and fully elastic, which is not true when looking at biological samples. However, it can be a good approximation (Carmichael et al., 2015).

The model has some deviations due to what kind of geometrical shape the tip of the intender has, varying between conical, parabolic and spherical. The applied force is a function of the indentation depth given by

$$F = \frac{4}{3}\frac{E}{(1-v^2)}R^{1/2}\delta_s^{3/2} \tag{5.1}$$

for a spherical tip (Rico et al., 2005; Sen et al., 2005; Brunner et al., 2006; Kuznetsova et al., 2007; Pelling et al., 2007; Carmichael et al., 2015) with R being the radius of the intender if the surface is flat (Darling et al., 2007). Note that there exist controversy in articles about the formulation of the Hertz model. (Vinckier and Semenza, 1998) and (JPKinstruments, n.d.) mentions the same formula when discussing the parabolic tip, which is understandable due to the similarity between the spherical and parabolic shape. (Radmacher, 1997) formulates the Hertz model for a conical intender as

$$F = \frac{2}{\pi}\frac{E}{(1-v^2)}\delta_s^2 tan\theta \tag{5.2}$$

where θ is the opening angle of the conical tip.

Calculating the force-indentation curve and fitting it with the Hertz model allows the estimation of Young's modulus, previously illustrated in figure 3.4. The value of E will be given by the best match between the two curves. One approach is to minimize the square of the error between AFM data, F_A, and the Hertzian response, F_H.

$$E = \sum_{i=0}^{n}(F_A^i - F_H^i)^2 \tag{5.3}$$

where n is the number of data points (Tripathy, 2005).

(Pelling et al., 2007) points out that it is more interesting to look at the relative changes in mechanical parameters rather than absolute values when discussing biological samples. Especially stiffness measurements are dependent on the same experimental conditions to be comparable (Haase and Pelling, 2015). (Pelling et al., 2007) proposed a normalized Hertz model showing the relative changes in Young's modulus given by

$$E^* = \frac{E_n}{E_0} = \frac{\frac{A_n\pi(1-v^2)}{2tan\theta}}{\frac{A_0\pi(1-v^2)}{2tan\theta}} = \frac{A_n}{A_0} \tag{5.4}$$

with E_n as the Young's modulus measured at time intervals (t) and E_0 as the value of E at time zero. This factors out the major unknowns such as tip geometry and Poisson ratio, but the assumption is that they are constant.

The Hertz model has led to the development of various other models, each of them made to identify different types of parameters. Some of these are the Tu model and the Chen model, see (Brunner et al., 2006).

5.2 The Sneddon Model

The Sneddon model has the same assumptions as the Hertz model and thus the same challenges. It is used as a more accurate model when the tip of the cantilever is conical-shaped instead of equation (5.2). The expression is given by (Guz et al., 2014) as

$$F = \frac{8}{3\pi} E \delta_s^2 tan\theta \tag{5.5}$$

The model is only applicable for moderate indentations on thick parts of cells. A correction to this model, called Bottom Effect Cone Corrected (BECC) model, has been proposed, taking into account the finite thickness h of the soft sample (Guzman et al., 2015):

$$F = \frac{8}{3\pi} E \delta_s^2 tan\theta \times \left\{ 1 + 1.7795 \frac{2tan\theta}{\pi^2} \frac{\delta_s}{h} + 50.67 tan^2\theta \frac{\delta_s^2}{h^2} \right\} \tag{5.6}$$

The BECC model can also incorporate a viscoelastic extension (Cartagena-Rivera et al., 2015).

5.3 Other Models

5.3.1 Brush model

This is an extension of the Hertz and Sneddon models that considers the cell to be covered by a brush layer, which is treated as a separate cellular structure. The model has not been elaborated due to few available sources. Nevertheless, it is an interesting approach used to measure elasticity and can be studied in more detail when the research becomes more extensive. See (Guz et al., 2014) for details and a comparison relative to Hertz and Sneddon.

5.3.2 Johnson-Kendall-Roberts (JKR) model

The JKR model is similar in form to Hertz and Sneddon, but not mentioned that often. Just like the Derjaguin-Muller-Toporov (DMT) model (Prokopovich and Perni, 2011), it takes adhesive forces into account, which is not the case with Hertz and Sneddon. Read more in (Barthel, 2008; Benitez and Toca-Herrera, 2014; Efremov et al., 2015).

5.3.3 Solid models

Solid models are applied to results from many experimental techniques, including AFM, but they are very simplified. There exist models for both elasticity (linear elastic solid model) and viscoelasticity (linear viscoelastic solid model). Details can be found in (Lim et al., 2006; Rodriguez et al., 2013).

Chapter 6

Viscoelasticity Calculations

Instead of looking at the cell as purely elastic, it can be interesting also to take the viscous contributions into account, as cells are indeed viscoelastic. Several articles have revealed that also viscoelastic properties can to serve as indicators of cell disease (Babahosseini et al., 2015).

Different experiments can be used to measure viscoelasticity. Common ones are stress relaxation and creep experiments in the time domain and oscillatory tests in the frequency domain (Kollmannsberger and Fabry, 2009).

There are various approaches in which viscoelastic behaviour can be described:

1. Extending elastic models into the time domain.
2. A differential representation leading to a linear differential equation, which uses assemblages of springs and dashpots as models.
3. An integral representation defining an integral equation derived from the Boltzmann superposition principle.
4. Power-law models
5. Frequency-dependent stress and strain representations

Some articles mention that it is possible to extend elastic models into the time domain to model viscoelasticity. How to this with the Hertz model is shown in section 6.1.

The integral and differential models are not sufficient to fully describe a biological material, even though many papers might give that impression. As pointed out in section 2.2.4 when introducing the concepts of stress relaxation and creep experiments, there is an assumption that the elastic and viscous responses will behave linearly. This is not the reality. Nevertheless, they can be used to derive constants that can be used as a basis for comparison and prediction (Vincent, 2012). The two models are described in the time-domain.

The most popular model for viscoelasticity, based on the number of articles using it, is the differential representation with springs and dashpots, see section 6.2 for extensive details. The integral approach is not that often used, but will be briefly described in 6.3.

Both power-law models and the frequency-dependent stress and strain representation use oscillatory measurements, are expressed as functions of frequency and use the frequency-dependent shear modulus. They are explained in sections 6.4 and 6.5, respectively. At the end of the chapter, some other viscoelastic models in existing literature are mentioned.

As previously accentuated, all the models assume that the cells behave as linear viscoelastic solids, which is only true for sufficiently small deformations.

6.1 Extended Hertz Model

By Laplace transformation and use of the correspondence principle, the Hertz model can be expressed in the time domain. See (Darling et al., 2006) for the derivation.

$$F = \frac{4}{3}\frac{E}{(1-v)}R^{1/2}\delta_s^{3/2}\left(1+\frac{\tau_\sigma-\tau_\varepsilon}{\tau_\varepsilon}e^{-t/\tau_\varepsilon}\right) \tag{6.1}$$

where τ_σ and τ_ε are the relaxation times under constant load and deformation, respectively.

6.2 Spring and Dashpot Models

(Benitez and Toca-Herrera, 2014) points out that while force-curves is the most common tool to obtain Young's modulus, force relaxation and creep experiments are starting to get popular in the AFM community because they are also able to deliver information about relaxation times and viscosities of the different cell parts. AFM force relaxation tests are conducted setting the vertical position of the cantilever constant. In creep compliance tests the cantilever's force is kept constant (Moreno-Flores et al., 2010).

Any arbitrary linear viscoelastic behaviour can be modelled with networks of springs and dashpots arranged in series or parallel (Kollmannsberger and Fabry, 2011). The models can be used to mimic the response of viscoelastic surfaces under interaction with the AFM tip (Lopez-Guerra and Solares, 2014). In this section, different spring-dashpot models will be described, starting off with the simplest combinations and then increasing the complexity. Some comments on parameter estimation are also included.

6.2.1 Linear Maxwell and Kelvin-Voigt models

Linear Maxwell and linear Kelvin-Voigt are examples of models constructed with springs and dashpots, which represents elastic and viscous components, respectively (Haase and Pelling, 2015). The springs and dashpots are described by elastic modulus E and viscosities μ (Lubliner, 1990). The springs obey Hooke's law, so that $E = \sigma_s/\varepsilon_s$, and in the dashpot the expression $\mu = \sigma_d/\dot{\varepsilon}_d$ is valid. The subscripts s and d corresponds to the experienced forces and deformations in the springs and dashpots.

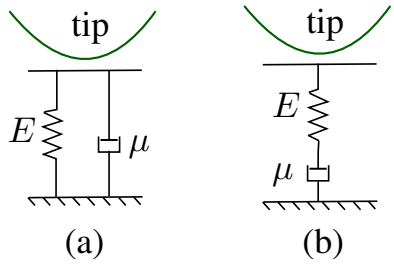

Figure 6.1: Linear Kelvin-Voigt(a) and Maxwell(b) models

The Kelvin-Voigt model uses a linear spring in parallel with a dashpot, see figure 6.1(a). This model can reproduce time-dependent creep compliance with high accuracy, but not stress relaxation (Solares, 2014). The surface lacks a spring that can accommodate the immediate force applied to it. Because of this, the single spring in the model does not have an instant response and only experiences compression until the parallel dashpot starts yielding (Lopez-Guerra and Solares, 2014).

In a creep experiment, the strain decays exponentially with a characteristic time constant $\tau = \mu/E$, so that

$$\varepsilon = \varepsilon_0 \exp(-t/\tau) \tag{6.2}$$

where ε_0 is the initial strain. See (Vincent, 2012) for deduction and details.

The differential equation describing the Kelvin-Voigt model is (Kelly, 2015):

$$\sigma = E\varepsilon + \mu\dot{\varepsilon} \tag{6.3}$$

The Maxwell model consists of the same two elements, a linear spring and a dashpot, but arranged in series (figure 6.1(b)). This model reproduces stress relaxation under constant strain, but not creep compliance (Vincent, 2012; Solares, 2014). During retraction of the cantilever tip in stress relaxation experiments, the sample experiences elastic recovery, but not viscous recovery due to the lacking mechanism in the dashpot to return to its original

position (Lopez-Guerra and Solares, 2014). In (Roylance, 2001) there is a deduction of the differential equation that describes the Maxwell model as

$$\dot{\varepsilon} = \frac{1}{E}\dot{\sigma} + \frac{\sigma}{\mu} \tag{6.4}$$

In a stress relaxation experiment, $\dot{\varepsilon} = 0$, which yield an equation similar to (6.2) given as

$$\sigma = \sigma_0 \text{exp}(-t/\tau) \tag{6.5}$$

The relaxation modulus, E_{rel}, is in turn

$$E_{rel}(t) = E\text{exp}(-t/\tau) \tag{6.6}$$

6.2.2 Standard linear solid model and Zener model

To be able to capture both stress relaxation and creep compliance, a combination of the Maxwell and Kelvin-Voigt models have been developed. It is called the Standard Linear Solid (SLS) model (Solares, 2014). When applied to normal stress, the strain creeps towards a limit, while, under constant strain stress relaxes towards a limit.

It seems to be consensus on how this model is defined and it can be viewed in figure 6.2 (Vincent, 2012; Lopez-Guerra and Solares, 2014; Carmichael et al., 2015; Haase and Pelling, 2015).

Here, the system relaxes through the dashpot located in the linear Maxwell arm, but the stress does not relax to zero as some of it remain stored in the parallel spring. It is denoted E_e because it provides an "equilibrium" (Roylance, 2001). This behaviour is more accurate than a total relaxation of the stress. As for the creep simulation, there is an immediate elastic response, which is missing in the linear Kelvin-Voigt model.

(Chester, 2012) provides the mathematical expression for the SLS model,

$$\dot{\varepsilon} = (E + E_e)^{-1}\left(\dot{\sigma} + \frac{E}{\mu}\sigma - \frac{EE_e}{\mu}\varepsilon\right) \tag{6.7}$$

In the case of stress relaxation, the relaxation modulus is similar to the one in the Maxwell model given by equation (6.6), but shifted upwards by an amount of E_e:

$$E_{rel}(t) = E_e + E\text{exp}(-t/\tau) \tag{6.8}$$

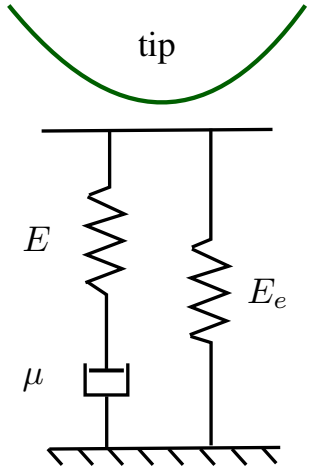

Figure 6.2: Standard linear solid (SLS) model

The drawbacks of the SLS model is that it can not reproduce multiple relaxation times (Lopez-Guerra and Solares, 2014). Due to this, other models have emerged. One of them is a series of linear Maxwell arms in parallel with an equilibrium spring to model multiple relaxation times, which can be viewed in figure 6.3. According to (Lopez-Guerra and Solares, 2014) this combination of springs and dashpots is called the Wiechert model and the number of Maxwell's arms corresponds to the number of relaxation times, which is important when molecular segments with different contributions have different lengths. Another version, however with only two Maxwell arms, is referred to as the Zener model in (Moreno-Flores et al., 2010). (Vincent, 2012) calls the Wiechert structure a "Generalized Maxwell model", but points out that any combination of multiple either Kelvin-Voigt or Maxwell elements (without mixing them) will obtain a spectrum of time characteristics. See section 6.2.3 for more details about these models.

Note that many articles describes a model they call Zener, but few agrees on the same structure. As previously commented, (Moreno-Flores et al., 2010) defines it as a Wiechert model with two Maxwell arms. (Carmichael et al., 2015) derives a version called fractional Zenar, which is similiar to the SLS model, but with the linear damper replaced by a fractional element. On the contrary, (Nobile et al., 2007; Moeendarbary and Harris, 2014; Zhu et al., 2014) claims that Zener and the SLS model is completely similar. This is mentioned to make the reader aware that there is not always consistency in the naming of different spring-dashpot models.

6.2.3 Wiechert/Generalized Maxwell model

Now, one of the more complex spring-dashpot models remains. Some authors call it the Wiechert model (Roylance, 2001; Machiraju et al., 2006; Lopez-Guerra and Solares, 2014), and others the Generalized Maxwell model (Vincent, 2012; Babahosseini et al.,

2015), but it has the same components.

As described in section 6.2.2, it consist of n Maxwell elements in parallel with a spring, see figure 6.3. If each of these have a different time constant, τ, the decay of stress will be spread over a longer period as a result of a broader spread of relaxation times.

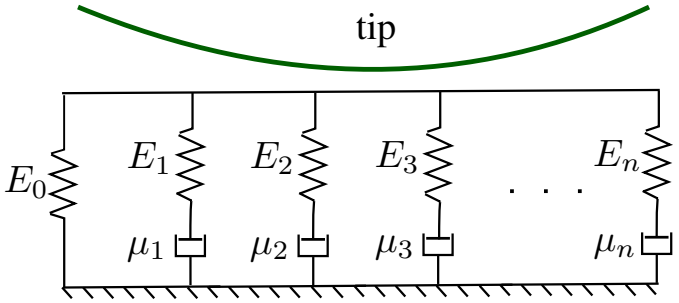

Figure 6.3: Generalized Maxwell model

If the Generalized Maxwell model is used to represent the cell surface, stress relaxation experiments are used. Creep experiments are being used in combination with a Generalized Kelvin-Voigt model. This is n Kelvin-Voigt elements in series with a spring, but there will not be provided details here, see (Haghighi-Yazdi and Lee-Sullivan, 2011) for this.

Deduction of the Generalized Maxwell model is retrieved from (Machiraju et al., 2006; Vincent, 2012). From equation (2.5) and (6.5) it can be seen that the expression for a single Maxwell element is

$$\sigma(t) = E \cdot \varepsilon \exp(-t/\tau) \tag{6.9}$$

For a number of Maxwell elements joined in parallel at the same strain, ε, the stress is

$$\sigma(t) = \varepsilon \sum^{n} E_{rel,n} \exp(-t/\tau_n) \tag{6.10}$$

where $E_{rel,n}$ and τ_n are the relaxation modulus and relaxation time of the nth element.

6.2.4 Parameter estimation in spring-dashpot models

By use of stress relaxation and creep experiments, and describing the cell surface through springs and dashpots, the time-varying strain and stress can be viewed. Estimation of the unknown parameters is possible in the corresponding differential equations. Take for instance equation (6.3) describing the Kelvin-Voigt model. Stress, $\sigma = F/A$, and strain,

$\varepsilon = \Delta l/l_o$, can be measured. If it is also possible to say something about the change in strain, $\dot{\varepsilon}$, by for example looking at the creep curve, there are only two unknown parameters left; E and μ.

It may be an idea sticking to the simpler models, with simpler differential equations, when doing parameter estimation with some compromise of accuracy. However, the same methods can be used on the SLS model, given in equation (6.7), by gathering the unkown parameters into one unknown parameter and then do parameter identification on this. For instance, $E/((E+E_e)\mu)$ kan be expressed as just k_1 and $(EE_e)/((E+E_e)\mu)$ as k_2.

An advantage with the spring-dashpot models is that the unknown parameters are linear.

6.3 The Integral Model

This approach is based on the Boltzmann superposition theory stating that the total response to a number of individual excitations is the sum of the responses that would have been generated by each excitation working alone. For example, $\sigma(\varepsilon_1 + \varepsilon_2) = \sigma(\varepsilon_1) + \sigma(\varepsilon_2)$. Also, previous actions in and on the material influence its present behaviour. To illustrate this, the total strain at time t is given by (Vincent, 2012; Roylance, 2001)

$$\varepsilon(t) = \int_{-\infty}^{t} J(t-\tau_n) d\sigma(\tau_n) \tag{6.11}$$

which is usually rewritten as

$$\varepsilon(t) = \int_{-\infty}^{t} J(t-\tau_n) \frac{d\sigma(\tau_n)}{d\tau_n} d\tau_n \tag{6.12}$$

Similarly for stress,

$$\sigma(t) = \int_{-\infty}^{t} E_{rel}(t-\tau_n) \frac{d\varepsilon(\tau_n)}{d\tau_n} d\tau_n \tag{6.13}$$

6.4 Power-law Models

According to (Kollmannsberger and Fabry, 2011), the spring and dashpot models are not sufficient for explaining the viscoelastic behaviour of cells because there is too large uncertainty in the fit parameters. Instead, power laws can describe tissue biomechanics.

Power-law model results are achieved from oscillatory tests and given as functions of frequency. As mentioned in section 2.2.3 there exist a frequency-dependent shear modulus, $G^*(\omega)$. It is either referred to as the complex shear modulus with a real and imaginary part (Lim et al., 2006; Bansod and Bursa, 2015; Hecht et al., 2015)

$$G^*(\omega) = G^{'} + iG^{''} \tag{6.14}$$

or the dynamic shear modulus (Hoffman and Crocker, 2009; Moeendarbary and Harris, 2014)

$$|G^*(\omega)| = \sqrt{(G'(\omega))^2 + (G''(\omega))^2} \tag{6.15}$$

In both cases $G^{'}$ is called the storage modulus and $G^{''}$ the loss modulus, representing the elastic and viscous responses.

From (Alcaraz et al., 2003):

> *"A straightforward and robust approach to characterize cell microrheology is by determining its complex shear modulus from oscillatory measurements over a wide frequency range. G^* is defined as the complex ratio in the frequency domain between the applied stress and the resulting strain. The real and imaginary parts of $G^*(\omega)$ account for the elastic energy stored and the frictional energy dissipated within the cell at different oscillatory frequencies. The ratio between the imaginary and real parts of $G^*(\omega)$ indicates the degree of solid- or liquidlike mechanical behaviour of the cell."*

There are different formulations of power-law models. The next sections will look at various representations. It is important to note that many of the articles used as sources on these models are not made specifically for AFM, but it is pointed out by (Alcaraz et al., 2003) that much of the work done on AFM is elasticity calculations and there exist little information on oscillatory mechanics probed with AFM. Which sources that uses power-law models in AFM context will be specified during the exposition.

6.4.1 Power-law structural damping model

The articles (Fabry et al., 2001; Alcaraz et al., 2003; Lim et al., 2006; Roca-Cusachs et al., 2006; Hiratsuka et al., 2009; Bansod and Bursa, 2015) describe a power-law structural damping model. Here, the complex shear modulus is defined as in equation (6.14) and extended to:

$$\begin{aligned} G^*(\omega) &= G^{'} + iG^{''} \\ &= G_0(\frac{\omega}{\omega_0})^{\alpha}(1 + i\eta)\Gamma(1-\alpha)cos(\frac{\pi\alpha}{2}) + i\omega\mu \end{aligned} \tag{6.16}$$

α is the power-law exponent and ω is the radian frequency $2\pi f$ (Fabry et al., 2001; Bansod and Bursa, 2015). η is the structural damping coefficient given by

$$\eta = G^{''}/G^{'} = tan(\alpha\pi/2) \tag{6.17}$$

G_0 and ω_0 are scaling factors for stiffness and frequency, respectively. μ is a viscosity material parameter and depend on bead-cell geometry, which is also the case with G_0. Γ is the gamma function with the properties $\Gamma(n) = (n-1)!$ and $\Gamma(x+1) = x\,\Gamma(x)$. It is defined for all complex numbers except non-positive integers.

(Alcaraz et al., 2003; Lim et al., 2006; Hiratsuka et al., 2009) use the power-law structural damping model with AFM measurements. Here, equation (6.16) is used as a fitting model for the calculated $G^*(\omega)$. The concept is to use an elasticity model like e.g. Hertz to relate the loading force and the indentation depth. Then, using the relation $G = E/2(1+v)$ to express G and next transform it to the frequency domain, an expression for a measurable $G^*(\omega)$ is obtained. A more detailed derivation is given in (Alcaraz et al., 2003). The result is (Roca-Cusachs et al., 2006):

$$G^*(\omega) = \frac{1-v}{4(R\delta_0)^{1/2}} \left[\frac{F(\omega)}{\delta(\omega)} - i\omega b(0) \right] \tag{6.18}$$

using the Hertz model. According to this equation, the frequency dependence of the cell mechanical response is included in the term in brackets, whereas the factor $(1-v)/(4(R\delta_0)^{1/2})$ accounts for the dependence on the tip geometry (Alcaraz et al., 2003). δ_0 is an operating indentation in which the indentation oscillations, $\delta(\omega)$, take place around. b is a drag factor depending on the height from the cell surface and expressed as $b(0)$ at contact. This value is possible to measure, see (Alcaraz et al., 2003; Hiratsuka et al., 2009) for details.

6.4.2 Power-law rheology

Another approach to power-laws is given by (Kollmannsberger and Fabry, 2011) and (Hecht et al., 2015) and they refer to it as power-law rheology without a specific name on the model. Only (Hecht et al., 2015) use it in combination with AFM while (Kollmannsberger and Fabry, 2011) reviews rheology of living cells in general.

The authors of the two articles does not have a completely similar end product, but agree on the definition of creep compliance in power law rheology, which is given by

$$J(t) = j_0 \cdot (\frac{t}{\tau_0})^{\beta} \tag{6.19}$$

The prefactor j_0 characterizes the material's compliance at t_0, giving $j_0 = J(t = t_0)$. Time is normalized by a timescale τ_0, which can be arbitrarily set to 1 s or any other convenient value. β is the power-law exponent with a value between 0 and 1, where $\beta = 0$ indicates a purely elastic solid and $\beta = 1$ a purely viscous fluid (Hecht et al., 2015). Changing τ_0 will not affect the value of β, leaving the system behaviour timescale invariant (Kollmannsberger and Fabry, 2011).

The rest of this section is divided into derivation of the complex modulus, which is given with some deviation in the two articles.

(Kollmannsberger and Fabry, 2011)
The complex modulus of the cell is defined by the Fourier-transformed displacement and force. Equation (6.19) then transforms to a power law with the same exponent β,

$$G^*(\omega) = \frac{1}{j_0}(i\omega\tau_0)^\beta \Gamma(1-\beta) \tag{6.20}$$

This is a very simple empirical relationship, as there is only one parameter that is free-fit: the power-law exponent β. Higher values of β point to a more fluid-like behaviour, while lower values to a more elastic behaviour (Hecht et al., 2015). At intermediate values of β, both elastic and viscous mechanisms coexist. Equations (6.19) and (6.20) are only valid in the limit of long timescales or low frequencies. See (Kollmannsberger and Fabry, 2011) for more details.

(Hecht et al., 2015)
Here, the authors have chosen to write the complex modulus with Young's modulus instead of the shear modulus. They both have the same properties as with loss and storage modulus, so $E^*(\omega)$ can be written as

$$E^*(\omega) = E^{'} + iE^{''} \tag{6.21}$$

The Young's modulus at t_0 is defined as $E_0 = 1/j_0$. From the parameters E_0 and β the storage and modulus can be expressed as

$$E^{'} = E_0\Gamma(\beta+1)cos(\beta\pi/2) \tag{6.22}$$
$$E^{''} = E_0\Gamma(\beta+1)sin(\beta\pi/2) \tag{6.23}$$

These two equations are related by a loss tangent $tan\,\theta = E^{''}/E^{'} = tan(\beta\pi/2)$. Notice the similarity to (6.17). Now, (6.21) can be rewritten to

$$E^* = E_0\Gamma(\beta+1)e^{\frac{\beta\pi}{2}i} \tag{6.24}$$

(Hecht et al., 2015) have used this to develop a new AFM technique to measure viscoelastic creep of live cells. They call it force clamp force mapping (FCFM), and the technique combines force-distance curves with an additional force clamp phase during the tip-sample contact.

6.4.3 Power-law model with dynamic shear modulus

Unlike the previous models explained there also exist a power-law model that is formulated using the dynamic shear modulus instead of the complex version, given by (Hoffman et al., 2006; Hoffman and Crocker, 2009). Note that neither of the articles is focused on AFM in particular, but rather cell mechanics in general.

In the review done on cell mechanics by (Hoffman and Crocker, 2009) it is pointed out that it has been difficult to compare the results of cell rheology measurements because

> *"Different labs would study different cell types and present their data in different forms, for example, as a creep function after applying a step stress, as a function of oscillation frequency, or in terms of springs and dashpots. Even with a given method, different labs might use tracers/probes with varying chemistry or size or make different assumptions to quantitatively interpret or calibrate their measurement. Of course, comparisons were still made, usually of the overall stiffness, and the many results were found to be discordant - reported stiffness values varied by orders of magnitude.*

However, with time it emerged some patterns, showing that the dynamic shear moduli could be described as a sum of two power laws (Hoffman et al., 2006):

$$\begin{aligned} G^{'}(\omega) &= Acos(\pi\beta/2)\omega^{\beta} + Bcos(3\pi/8)\omega^{\beta} \\ G^{''}(\omega) &= Asin(\pi\beta/2)\omega^{\beta} + Bsin(3\pi/8)\omega^{\beta} \\ |G^{*}(\omega)|^2 &= G^{'}(\omega)^2 + G^{''}(\omega)^2 \end{aligned} \tag{6.25}$$

β varies with different experiments and ranges between 0.1 and 0.3 (Hoffman and Crocker, 2009).

They also point out that an important consideration when fitting data is that either both $G^{'}(\omega)$ and $G^{''}(\omega)$ should be fit or $|G^{*}(\omega)|$ should be fit.

6.5 Frequency-dependent Stress and Strain

This section discusses how to represent stress and strain when the cell is exposed to oscillations from AFM. As in the power-laws, the storage modulus, $G^{'}$, and loss modulus, $G^{''}$, are being used to express the behaviour.

There is disagreement in the definition of stress and strain functions during dynamic loading experiments, but some of them will be rendered here.

(Roylance, 2001)

If the origin along the time axis is selected to coincide with a time at which the strain passes through its maximum, the strain and stress functions can be written as

$$\varepsilon = \varepsilon_0 \cos(wt) \tag{6.26}$$

$$\sigma = \sigma_0 \cos(wt + \varphi) \tag{6.27}$$

wherre φ is the phase lag. The stress function can be written as a complex quantity

$$\sigma^* = \sigma_0^{'} \cos(wt) + i\, \sigma_0^{''} \sin(wt) \tag{6.28}$$

and the following relations hold:

$$\tan \varphi = \sigma_0^{''} / \sigma_0^{'} \tag{6.29}$$

$$|\sigma^*| = \sigma_0 = \sqrt{(\sigma_0^{'})^2 + (\sigma_0^{''})^2} \tag{6.30}$$

$$\sigma_0^{'} = \sigma_0 \cos \varphi \tag{6.31}$$

$$\sigma_0^{''} = \sigma_0 \sin \varphi \tag{6.32}$$

$$G' = \sigma_0^{'} / \varepsilon_0 \tag{6.33}$$

$$G'' = \sigma_0^{''} / \varepsilon_0 \tag{6.34}$$

(Vincent, 2012)

$$\sigma_0 = \varepsilon_0\, G^* sin(\omega t + \varphi) \tag{6.35}$$

which can be extended to

$$\sigma_0 = \varepsilon_0 (G^* cos\varphi) sin\,\omega t + \varepsilon_0 (G^* sin\varphi) cos\,\omega t = \varepsilon_0 G^{'} sin\,\omega t + \varepsilon_0 G^{''} cos\,\omega t \quad (6.36)$$

(Moeendarbary and Harris, 2014)

$$\sigma = \sigma_0 sin(\omega t + \varphi) = G^{'}(\omega) sin(\omega t) + G^{''}(\omega) cos(\omega t) \quad (6.37)$$

$$|G^*| = \sqrt{(G')^2 + (G'')^2} \quad (6.38)$$

The following properties are valid for pure elastic and viscous materials:

Pure elastic materials:	$\varphi = 0$	$G^{'} = G$	$G^{''} = 0$	$\|G^*\| = G$
Pure viscous materials:	$\varphi = \pi/2$	$G^{'} = 0$	$G^{''} = \mu\omega$	$\|G^*\| = \mu\omega$

6.6 Other Models

6.6.1 Liquid drop models

The liquid drop models are also referred to as cortical shell-liquid core models. They were developed for micropipette aspiration and optical tweezers and views the suspended cell or its parts as a deformable material with certain continuous material properties. Variations are Newtonian, compound Newtonian, shear thinning and Maxwell liquid drop models. Read more in (Lim et al., 2006; Rodriguez et al., 2013; Bansod and Bursa, 2015).

6.6.2 Tensegrity model

The tensegrity model is derived from the micro/nanostructural approach and therefore not elaborated. It assumes that the intermediate and actin filaments in the cytoskeleton carry a stabilizing tensile stress (”prestress”) that is balanced by internal microtubules and extracellular adhesions. See (Sultan et al., 2004; Moeendarbary and Harris, 2014; Haase and Pelling, 2015) for details.

6.6.3 Biphasic model

This is a model developed for cytointender that treats the cytoplasm as both solid and fluid-like. Different variations are biphasic poroelastic and poro-viscoelastic models. See more in (Lim et al., 2006; Moeendarbary and Harris, 2014; Bansod and Bursa, 2015).

Chapter 7

Current Challenges

The work on improving AFM is in constant progress. One of the main objectives is to further increase the scanning speed. (Cartagena-Rivera et al., 2015) recently introduced a new technique that supposedly boosts the speed of imaging cells in dynamic AFM mode by at least one order of magnitude. This is done using the cantilever mean deflection as the feedback signal instead of the amplitude.

There are also challenges related to the mechanical models of cells, as it will be pointed out in this chapter.

7.1 Inaccuracy of Models

All models described in this thesis assume that the probed cell shows linear behaviour. This is not the reality and leads to reduced accuracy in their description of cell mechanics.

7.1.1 Elastic models

In the mentioned techniques in chapter 5 when doing elasticity calculations, the indentation is limited to approximately 10-15 % of the cell height. This excludes information that may lay deeper into the cell that potentially can provide information for mechanical diagnosis of disease (Carmichael et al., 2015).

In addition, the Hertz and Sneddon models assume that cells are homogenous, but in reality, they are heterogeneous with the result that the organelles have different contributions to cell elasticity. One example is that the nucleus is known to be stiffer than the cytoplasmic portion of the cell (Moeendarbary and Harris, 2014) and that the elastic moduli of

purified filament networks are orders of magnitude lower than whole-cell measurements (Haase and Pelling, 2015). It is important to note that there are different areas of rigidity within one cell and that the Young's modulus depends on the depth of the probe penetration.

A solution to these challenges is to use the Hertz and Sneddon models as approximations and keep in mind that they are not an exact representation of the reality. Simultaneously, it is important to continue the search for better models.

Computing the point of contact between the cantilever tip and the sample is difficult with the Hertz model because of uncertainties in the tip geometry and adhesive influencing forces (Heinz and Hoh, 1999; MacKintosh and Schmidt, 1999). A possible solution to this is to automate the contact point (CP) selection. The CP is not known before an experiment starts, but have an impact on the correct assessment of mechanical properties. See (Chang et al., 2014) for more details on this topic and their proposed method to improve current techniques.

7.1.2 Viscoelastic models

At low applied forces by AFM and small resulting deformations, cells present linear elastic behaviour. At higher forces, a viscoelastic behaviour is observed (Haase and Pelling, 2015). However, the existing viscoelastic models are not complex enough to accurately describe what happens in the cell at these high forces. As (Vincent, 2012) points out about viscoelastic models:

> *"their mathematical representations rely on linearity of response of both elastic and viscous components. This is normally considered to be attainable only at strains of less (usually much less) than 0.01, but nearly all biological materials are not only nonlinear in response, but normally function at high and extremely high (0.5+) strains. The models for viscoelasticity are not valid under these conditions. This is a severe limitation and one that is not commonly recognised. Thus much work on artificial and natural polymers is of dubious value, because it applies linear, small-strain models to nonlinear, large-strain materials. That such data may well often be internally consistent is no argument for the acceptance of the linear interpretation; it may merely be coincidence. The mathematics of viscoelasticity at large strains remains to be worked out."*

7.2 Finding a Universal Model

Although the study of cell mechanics has developed fast in recent decades, there still does not exist a complete theoretical description of cell mechanics that is both time-dependent and predictive (Pelling and Horton, 2008). (Haase and Pelling, 2015) says that an all-encompassing theory of cell rheology must rely on a coarse-grained picture of the cell and

that a potentially full range description of cell behaviour will require a complex nonlinear model.

The ultimate result would be to find a single model general enough to describe accurately the response of (almost) any cell type to any applied mechanical stimulus. Because most cell types have similar mechanical parts, a universal model could be used by changing material constants and parameters for each cell type (Rodriguez et al., 2013).

7.3 Description of Nonlinear Behaviour

A topic that hasn't been covered in this thesis is a description of the nonlinear behaviour of cells. The models reviewed here can only describe the linear response in the cells when applied to small external forces. To accurately describe cell's true mechanical behaviour, models that can capture nonlinear response may be necessary.

(Lopez-Guerra and Solares, 2014) discuss a standard nonlinear solid (SNLS) model that can say something about nonlinear behaviour when the AFM tip is in contact with the sample.

(Carmichael et al., 2015) address the problem of using linear models to describe cells and propose a fractional model to allow for a non-integer time-derivative relationship between stress and strain. See also (Kollmannsberger and Fabry, 2011) for a discussion about nonlinear mechanical properties of cells.

Chapter 8

Summary

This thesis has reviewed existing mechanical models describing biological cells. The two main categories of models are elastic models and viscoelastic models. The former is not a true representation of how the cell will respond to applied forces, but a good approximation to find Young's modulus. The most important model is based on Hertz theory of elastic contact, while variations of this are used in cases where the geometry of the cantilever tip is not spherical or when adhesive forces can not be neglected.

By looking at cells as an elastic material, only the applied stress and the resulting strain are taken into account. Viscoelastic models are better descriptions of the actual response of cells because they also acknowledge that cell's behaviour varies with time. The models that describe viscoelastic behaviour are more diverse than the elastic models. Out of these, spring-dashpot models are most frequently used. They mimic the response of the cell surface by combinations of springs and dashpots and can be described by differential equations. Less used approaches are the integral model and extension of the Hertz model into the time domain.

If dynamic experiments are performed, stress and strain will be functions of frequency rather than time. Here, different power-law models are used to describe viscoelastic behaviour, but the literature is inconsistent on the best formulation.

Parameter estimation is possible to some extent on these models. In elastic models, the parameter estimation is mainly done through curve fitting of force-distance curves to known models of elastic contact. In the viscoelastic models, the most straightforward choice is performing it on the spring-dashpot models, as they are described by differential equations with linear unknown parameters.

Chapter 9

Conclusion

There has already been conducted a lot of work on cell mechanics with AFM. The intention of this thesis was to gather the existing information and present an overview. The result is not a complete manual, but a contribution to a deeper understanding of cell mechanics using AFM.

Due to limited prior knowledge in the field of biology and cell mechanics, it has been a challenge to evaluate the accuracy of the material investigated. The sources used in this thesis are mainly published articles, the majority of them from journals, but also some conference papers. A few books and master theses are also cited. Even if this material can be assumed peer-reviewed in some or greater extent, there is no guarantee that all their content is reliable. The solution has been to trust articles that have acquired a lot of citations from other authors, but ignore this to some extent when dealing with more recent publications. If there have been disagreements among different papers on for example formulas, the opinion of the majority has been chosen. Alternatively, details of the various approaches have been explained. However, to limit material that may not interest the reader, the relevant articles have often been referred to for more detailed explanations and derivations.

Because some of the articles cited throughout the text are used for definitions and background theory, it is desirable to highlight the most relevant ones. In the Appendix, there is a table with a list of articles worth looking into if the reader is interested in gaining a better understanding of different topics. The table shows what kind of material the article covers, divided into AFM, calculation of Young's modulus (Young), spring-dashpot models (S-D) and power-law models (P-L). In addition, two columns show if the article is a review article or not, and how much it has contributed to this thesis, named "Rev." and "Contr." respectively.

In addition to the summary of cell mechanics presented by this thesis, the bibliography and table in the Appendix are the main contributions to the further work on parameter

estimation in these models.

9.1 Recommendations For Further Work

It would be interesting to investigate models that can describe the nonlinear behaviour of cells. This will be a better representation of how cells respond to applied forces. The models presented in this review assumes linear response of both elastic and viscous elements in the cell, and this is not an authentic portrayal of the reality.

However, with the goal of doing parameter estimation, the models that assume linear behaviour is the best starting point. Especially spring-dashpot representations of the cell surface look promising. Sources that seems interesting to investigate are (Kim et al., 2004; Tripathy, 2005; Yuya et al., 2008).

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Appendix

Table of Relevant Articles

The signs in the table indicates

✓ yes

∼ some

× no

Article	AFM	Young	S-D	P-L	Rev.	Contr.
(Abramovitch et al., 2007)	✓	×	×	×	×	✓
(Alcaraz et al., 2003)	∼	∼	×	✓	×	✓
(Babahosseini et al., 2015)	✓	✓	∼	×	×	∼
(Benitez and Toca-Herrera, 2014)	✓	✓	×	×	✓	✓
(Bansod and Bursa, 2015)	×	×	∼	✓	✓	∼
(Brunner et al., 2006)	∼	✓	×	×	×	∼
(Butt et al., 2005)	✓	✓	×	×	✓	∼
(Capella and Dietler, 1999)	✓	✓	×	×	✓	∼
(Carmichael et al., 2015)	∼	∼	✓	×	×	∼
(Cartagena-Rivera et al., 2015)	✓	∼	∼	×	×	∼
(Fabry et al., 2001)	×	×	×	✓	×	∼
(Fairbairn and Moheimani, 2013)	✓	×	×	×	×	∼
(Guz et al., 2014)	∼	✓	×	×	×	∼
(Haase and Pelling, 2015)	✓	∼	✓	✓	✓	✓
(Hecht et al., 2015)	✓	∼	×	✓	×	✓

Article	AFM	Young	S-D	P-L	Rev.	Contr.
(Heinz and Hoh, 1999)	✓	✓	×	×	✓	✓
(Hoffman et al., 2006)	×	×	×	✓	×	∼
(Hoffman and Crocker, 2009)	∼	×	×	✓	✓	✓
(JPKinstruments, n.d.)	✓	✓	×	×	×	✓
(Kollmannsberger and Fabry, 2009)	×	×	×	✓	×	∼
(Kollmannsberger and Fabry, 2011)	∼	×	∼	✓	✓	✓
(Kurland et al., 2012)	✓	✓	×	×	✓	∼
(Kuznetsova et al., 2007)	✓	✓	×	×	✓	∼
(Lim et al., 2006)	∼	∼	∼	✓	✓	✓
(Lopez-Guerra and Solares, 2014)	✓	×	✓	✓	×	✓
(Lubliner, 1990)	×	∼	✓	×	×	∼
(Moeendarbary and Harris, 2014)	✓	×	✓	✓	✓	✓
(Moreno-Flores et al., 2010)	✓	∼	✓	×	×	∼
(Pelling et al., 2007)	✓	✓	×	×	✓	✓
(Rabinovich et al., 2005)	✓	✓	×	×	×	∼
(Rodriguez et al., 2013)	∼	∼	×	✓	✓	✓
(Roylance, 2001)	×	∼	✓	∼	×	✓
(Sokolov, 2007)	✓	✓	×	×	✓	✓
(Solares, 2014)	✓	×	✓	×	×	∼
(Vincent, 2012)	×	✓	✓	✓	×	✓
(Vinckier and Semenza, 1998)	✓	✓	×	×	✓	∼