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abstract

This chapter gives an overview of recent advances in the field of cell mechanics. Mechanical cues influence many biological processes and a wide range of stiffnesses is present in different parts of the body. Powerful new experimental tools have expanded our understanding of constitutive processes at a single cell level. In particular, a combination of imaging and micro-fabrication techniques provide valuable insights into the differential cellular response to extracellular stiffness on a subcellular level.

\footnote{This chapter is based on: H. van Hoorn and T. Schmidt, A closer look at cellular forces - inversion improves resolution, to be submitted}
1.1 Mechanics matters

The human body continually applies deformative stresses and strains while performing a multitude of functions \([1-5]\). Deformation takes place on a range of length scales and stiffnesses. Blood is pumped through the vasculature, muscles are flexed in sarcomeric multicellular structures, and single cells (such as fibroblasts and neutrophils) exert significant forces on the extracellular matrix (ECM) while performing their task. In order to properly function at these different length scales, biological matter exhibits a great variety in mechanical characteristics.

The mechanics in such processes has been quantified in a wide range of previous studies. For example, endothelial cells lining blood vessels need to withstand the significant shear flow of 0.1-7 Pa throughout the vasculature \([5]\). An aggregate of approximately 500 contracting cardiomyocytes can produce periodic forces of \(\sim 10 \mu \text{N} \) with a stress accumulation \(\sim 1 \text{kPa} \) \([6]\). And individual neutrophils exert nN forces on the ECM to invade \([7]\) and adapt their membrane tension to phagocytose \([8]\). The mechanical behavior of cell aggregates, tissues and single cells is vital to their functioning in an organism. To gain a quantitative fundamental understanding of cell mechanics, we view a single cell as the basic building block. We aim to measure and understand how constitutive cellular processes contribute to cellular deformation and force exertion.

This biophysical behavior in turn expands our understanding of a wide range of biological functions.

Several crucial processes in cell biology have been shown to depend on mechanical cues. Stem cell differentiation \([9-11]\), cell migration \([12, 13]\) and cancer progression \([14, 15]\) have been directly linked to stiffness, force exertion and deformation. At a single cell level, contractile forces are typically exerted in the order of 1 - 100 nN at a single adhesion site \([16-19]\). All higher order structures making up tissues and other multi-cellular systems, are comprised of these basic building blocks. Constitutive cell mechanics is given by a cell’s ability to deform and exert forces in relation to intracellular protein localization and activation.

Still, the relevance of quantified cell mechanics to its biological niche needs to be taken into account. Therefore, we first discuss the variation of stiffness throughout an organism providing a framework for the relevance of extracellular stiffness. Next, we discuss recently developed methods to probe cell mechanics. Experimental approaches have been developed on a global or local scale that measure cellular mechanics ei-
ther through active perturbation or passive observation. We then discuss possible mechanical mechanisms that can influence biological behaviour and how cellular force exertion and deformation can be inferred from biological processes. Finally, we make a case that high-resolution imaging combined with new technology probing mechanics can provide answers to many crucial biophysical questions.

1.2 Stiffness varies

To properly compare the stiffness in the body, it must be described by a common variable. While a variety of moduli have been used in prior studies, we transferred all previous results to the common stiffness characteristic denoted by the Young’s modulus \[ E \]. This modulus describes a linear response between applied stress and strain thus giving a measure for stiffness, as given in equation (1.1). When studies reported a shear modulus, we directly translated this into a Young’s modulus assuming incompressability of the material (i.e. a Poisson ratio \( \nu \) of 0.5 with \( E = 2(1 + \nu)G \)). To enable comparison between various results, we consistently denoted here the stiffness by the Young’s modulus or an equivalent measure.

\[
\sigma = E \cdot \varepsilon
\]  

(1.1)

Diversity in the mechanical environment of a cell is best described by quantification of the stiffness. Next to the important assumption of linear elasticity, another difficulty in interpreting results is the length scale at which stiffness is probed. This is especially important since the length scale at which cells probe their environment remains a subject of debate \[ 17, 21, 22 \]. We will discuss this in section 1.5. Furthermore, the stiffness found in vivo at various lengthscales has proven to vary by several orders of magnitude \[ 23 \].

The length scale that is probed depends on the technique that is used. In previous studies, excised organs or reconstituted organ systems were probed either by a nano-scale indenter (typically using a probe with an Atomic Force Microscope - AFM) at a very local scale. The curvature of an AFM-tip is \( \sim 10-100 \) nm. A larger \( \sim 1-100 \) µm micron-sized probe has also been used to measure stiffness, as well as an optical cell stretcher \[ 24 \], to measure stiffness. In some studies, a macro-scale measurement was performed, typically using a rheometer, a tensile test apparatus or a
macro-indenter. We collected the results from measurements at different length scales and methods and discuss variations among different data sets - if they were observed - and relate them to length scale and/or experimental technique. Overall, we assumed linear elasticity, a similar and homogeneous stiffness for the different tissues in mammals and an identical local and global stiffness. It should be noted that for large deformations, simple linear elasticity does not hold for many biological networks [25, 26]. With these considerations in mind, we collected experimental data to show the diversity of stiffness in a (human) body.

Figure 1.1
Stiffness varies throughout the human body. Stiffness is quantified through the Young’s modulus and spans multiple orders of magnitude throughout the human body, as probed by experiments over various length scales. ²

Figure 1.1 shows an overview of the wide range of measured stiffnesses throughout the body (see also recent reviews [5, 27, 28]). The softest region is the brain, where stiffnesses ranging from 0.1 to 3 kPa have been reported [29, 32]. Interestingly, these measurements were mostly performed in macro-scale experiments that showed a relatively higher

²Image adapted from stock photo at www.freepik.com
stiffness. The only microscale AFM indentation study \cite{29} showed a stiffness of the rat hippocampus of 0.1-0.4 kPa. The stiffness of brain tissue at a smaller length scale may thus be even smaller, though this is technically a difficult experiment to perform.

Both macroscale rheometer measurements on freshly excised liver \cite{33} and microscale measurements on the glomerular capsule of the kidney \cite{34} showed a similar stiffness range of 1-3 kPa. Fat has been reported to have a stiffness of 3 kPa \cite{11}. In another study the stiffness of fat tissue has been quantified only by means of ultrasonic measurement on a macroscale \cite{35} and was found to be (20±10) kPa. The broadest interpretation of the stiffness of fat is thus a range of 3-20 kPa. Similar difficulties arose when looking for the stiffness of lungs \cite{36} and the endothelium \cite{37}, where values ranging from 1 to 100 kPa could be found, but were not always clear in their interpretation.

More experimental data is available for the stiffness of muscle tissue and cells. Excised mouse muscle showed a stiffness of (12±4) kPa \cite{38}. AFM indentations on (skeletal) muscle cells showed a larger range of stiffnesses of 10-40 kPa \cite{37,39}. One of these studies also addressed the stiffness of cardiac muscle, which was found to be 100 kPa \cite{37}. Another study which measured the stiffness of excised heart found the Young’s modulus to be (18±2) kPa \cite{40}. From this we conclude that muscle stiffness spans a wide range of 10-100 kPa.

Stiffness measurements of cartilage (from human tissue) showed a stiffness of 20-40 kPa, using both micropipette aspiration and AFM indentation techniques \cite{23,41}. Variations in these results can largely be explained by a difference in microscale versus nanoscale stiffness. One previous study \cite{23} showed that the nano-indentation results on mouse tissue probed the individual collagen fibrils. In the same study, however, microscale indentations could not resolve the fibrils and yielded very different results, where the large scale stiffness was 2 orders of magnitude larger. The nanoscale indentation results corresponded well to the previous measurements. We thus conclude that a reasonable estimate for the stiffness of cartilage is 20-40 kPa.

Through nanoscale AFM indentations, collagenous (not calcified-) bone was found to be (27±10) kPa \cite{9}. Global stiffness measures (tensile tests) on fibroblast-populated reconstituted matrices showed that areolar tissue could obtain stiffnesses of 60-400 kPa \cite{42,43}. Rat arteries showed an even larger stiffness of 100-1500 kPa \cite{44}. The stiffness of calcified
bone was determined to be much larger still, with stiffnesses measured in the order of GPa \([6, 27]\).

Throughout an organism a huge variety in stiffness is present. Without calcified bone, biological functionality is carried out over 5 orders of magnitude of stiffness. Stiffness is likely an emergence of evolution through diversification of biological material. Further evolution undoubtedly also guided functional responses to this diversification of stiffness. Biological matter has accordingly developed a coupling between biological and mechanical behavior at a cellular level. Understanding of the biological-mechanical coupling in the function of living matter is therefore crucial when dissecting many of life’s processes. Precise tools to control- and measure the mechanics of cells are vital in this research.

### 1.3 Techniques advance

For a quantitative understanding of cell mechanics we need to probe forces and measure deformations while monitoring relevant biological processes. In recent years, many technical steps forward have been taken that enable the accurate measurement of cellular forces and deformations. Active perturbation of cells yields new information about the outside-in coupling and response to a physical stimulus. This stimulus is often physiologically relevant, such as the deformation of heart muscle cells during the beating of a heart or the active deformation that expands lungs during breathing. In other processes, it is interesting to observe the inside-out coupling of a cell through a passive measurement. One can observe the local movement of vesicles, beads or fluorescently labeled proteins inside a cell, for instance. The extracellular deformation caused by a cell also provides important information in processes such as cancer cell migration to form metastatic sites. Figure 1.2 gives an impression of techniques that actively deform or passively quantify cell mechanics on a local or global cellular scale.

#### 1.3.1 Active deformation

Techniques actively probing cell mechanics include the application of atomic force microscopy (AFM), magentic tweezers (MTs) and a substrate stretcher \([5, 45]\). Local stiffness mapping of fibroblasts on substrates of different rigidities with an AFM showed that the stiffness of
1.3 Techniques advance

fibroblasts corresponded to the stiffness of their substrate up to a stiffness of 20 kPa [46]. Using MTs, it was possible to quantify the dynamic stiffness moduli of the cellular membrane [47]. These exciting developments allow one to measure local mechanical characteristics by actively perturbing a cell.

Since the intracellular organization is comprised of many components, it is also interesting to examine the cell-wide response to perturbation. An important and physiologically relevant perturbation is the physical stretching of whole cells through the ECM. Physical cell stretching occurs, for instance, when muscle cells contract or lungs expand. Several setups have been designed that enable the deformation of cells on a substrate [48–50]. Cells can also be stretched by trapping in a focused optical beam [24], enabling mechanical characterization of cells in suspension. The effect of disease on the force exertion characteristics by muscle cells is often quantified using dynamic force transducers, for instance to elucidate the effect of hypoxia [51].

In recent years, whole-cell active deformation has provided many valuable insights. Global alignment of the actin cytoskeleton was shown to occur when cells were stretched at mHz frequencies [49]. Organ level functions of a lung were even obtained by actively stretching a layered device with different cell types by mimicking functional alveoli [50]. Cell division, which is guided through a massive re-organization of the microtubule network, was shown to occur preferentially in the direction in which cells were stretched [52]. And a clue for a local mechanosensory mechanism was provided by global stretching of an intact cellular cytoskeleton that showed stretch-dependent phosphorylation of the putative mechanosensor p130Cas [53]. By globally deforming a whole cell on a stretchable membrane, the response of a cell can thus yield important new insights in the relation between active deformation and biological function.

1.3.2 Passive cell mechanics

Instead of actively deforming a cell, much can be learned from passively observing cell mechanics. Several intriguing techniques have been developed, among which the incorporation of submicron-particles into cells [54]. By performing tracking and (nano-)rheology experiments mechanical properties of cells are quantified. Using this technique, the role of the keratin intermediate filament network was investigated in determining
Figure 1.2
Approaches to probing Cell Mechanics. Active probing is done on a local scale by force spectroscopy techniques such as atomic force microscopy, magnetic tweezers or optical tweezers. Global active cellular deformation is achieved by stretching a substrate to which cells are attached. Passive- and local mechanics is probed by tracking beads or through fluorescence methods. On a global scale, cell mechanics is quantified by traction force microscopy or micropillars with which one can measure cellular force exertion.

subcellular stiffness [55]. In fibroblasts, the effect of actomyosin contractility on intracellular stiffness was measured [56]. Using a dual-labeling technique it was shown that stretch of a single talin molecule was dependent on myosin activity [57]. A FRET-based intracellular force sensor was designed by attaching to fluorophores onto either side of a spider-silk protein, for which force and distance were calibrated [58]. These techniques provide information on the local intracellular mechanics through
1.3 Techniques advance

an optical readout.

Instead of locally probing cell mechanics, passive measurement of how cells deform their environment is a topic of increasing interest. This approach has been the main focus of our research. In such studies mechanics is typically probed passively and on a cell-wide scale. Cell-wide force exertion was first quantified by tracking the wrinkles on a stretchable silicon substrate [59, 60]. This technique was adapted to include fluorescent beads [61] or regular patterns [16, 62] that could track the deformation field caused by a cell with higher accuracy and was termed traction force microscopy (TFM). This technique can yield a high-resolution force map [63] and was recently expanded to also quantify the out-of-plane force exerted locally at a site of adhesion [64]. TFM has provided many important insights in the way cells deform their environment and forces are transmitted. However, the effect of a varying extracellular stiffness remains difficult to investigate, since changing the stiffness of a substrate used in TFM invariably changes the local molecular architecture (i.e. connectivity, pore-size and active groups) of the substrate. Through this disadvantage, TFM cannot decouple the global extracellular stiffness and the local adhesion stoichiometry.

1.3.3 Micropillars

After the development of TFM, micropillar substrates were developed [65, 66]. With etching techniques on silicon wafers, a template for replica-molding with Poly(DiMethyl-)Siloxane (PDMS) was constructed [67, 68]. PDMS can be activated through oxidation which enables the direct binding of ECM proteins onto the surface. When the micropillars are functionalized through micro-contact printing [69] and the remaining PDMS is passivated, cells only attach to the very tops of the micropillars. Since cells mainly exert in-plane forces, this makes the pillar force-deflection relationship relatively easy to solve. Calculating back from a continuous substrate strain field to local force exertion sites (as is needed for TFM) is possible, but not straightforward [62]. Furthermore, variation in etch depth and thus pillar height provides direct control over the bending modulus of a single pillar and the global extracellular stiffness.

By measuring the deflections imposed onto micrometer-sized posts, the force a cell locally exerts is directly quantified. A disadvantage to micropillars relative to TFM is that the substrate is no longer continuous since the individual pillars need space to deform. Advantages to micropil-
lars are the more straightforward interpretation of the force-deflection relationship and the possibility to change the global extracellular stiffness (i.e. the pillar bending modulus) without changing the local site of attachment. Increasing the micropillar height decreases the global stiffness, while decreasing the height increases the stiffness.

The typical approximation for the force-deflection relationship is given by the well-known Euler-Bernoulli beam theory \[ \text{(20)} \], as given for a circular beam in equation \( \text{(1.2)} \). The force \( F \) relates to the pillar deflection \( \delta \) through the bulk material stiffness \( E \), the pillar diameter \( d \) and pillar height \( h \). Both diameter and height greatly influence the bending modulus through a fourth power and third power, respectively. The diameter of our pillars was 1 or 2 \( \mu \)m with a center-to-center distance of 2 or 4 \( \mu \)m, respectively. The range of diameter and spacing is limited since the dimensions need to be large enough to be accurately engineered and its deflection needs to be detected, while it needs to be small enough to be able to probe the force exerted by a cell at multiple locations. Importantly, the local stiffness \( E \) does not need to change, while the cell-wide global stiffness does change, decoupling the local extracellular environment from the global stiffness. In the current debate on whether cellular mechanosensing takes place on a local \[ \text{[21, 22, 70]} \] or cell-wide global scale \[ \text{[11, 17]} \], decoupling of these parameters can provide important insights.

\[
F = \frac{3\pi}{64} \cdot E \cdot \frac{d^4}{h^3} \cdot \delta \tag{1.2}
\]

Micropillar arrays have been used in a variety of ways since their development. It has been shown that asymmetric micropillars guide cell migration \[ \text{[71]} \], heart muscle cells have been suspended between pillars to examine their mechanical function \[ \text{[72]} \] and individual micropillars have been actively deflected using a magnetic probe to exert a local force on a cell \[ \text{[73]} \]. It has even been shown that stem cell differentiation depends on the global stiffness of the cellular environment as it is changed by pillar height \[ \text{[10, 68]} \] The advent of micropillars, and microstructured materials in general, has much promise to elucidate many of the outstanding questions in cell mechanics. However, to directly relate local cellular mechanics to biology, high-resolution optical microscopy must be possible simultaneously.
1.4 Combining imaging and mechanics

Next to cell mechanics techniques, optical microscopy has undergone a transformation as well in recent years. Breakthroughs were first made in single molecule spectroscopy \cite{74} and sub-diffraction limit \cite{75} localization of the intensity profile emitted by single molecules in live cells \cite{76}, enabling the localization of single fluorescent molecules. By localizing many molecules in rapid succession, super-resolution microscopy was developed less than a decade ago. Simultaneously, STochastic Optical Reconstruction Microscopy (STORM) \cite{77}, PhotoActivated Localization Microscopy (PALM) \cite{78} and fluorescence PhotoActivation Localization Microscopy (fPALM) \cite{79} were developed, all adhering to the same principle. Rapid switching of either fluorescent dyes or photo-convertible fluorophores provides a reconstruction of a labeled structure with a resolution better than the fundamental diffraction limit \cite{75}. STORM microscopy was simplified by introducing a reducing agent and by using standard fluorescent dyes \cite{80,81}. This innovation was termed direct STORM (dSTORM) and made the experiment easier to perform.

Super-resolution techniques can answer many open questions in cell mechanics. Processes in cell mechanics often take place beyond the diffraction limit through local molecular interactions. Especially when we consider mechanosensory mechanisms (further elaborated on in section 1.5) where a single or a few molecules localize differentially depending on mechanical features (e.g. extracellular stiffness). To enable the combination of the measurement of cell mechanics and single-molecule microscopy we needed to advance current techniques.

Limiting in the detection of single molecules is the amount of light one can detect. The localization precision of a molecule scales with the inverse square-root of the number of photons observed \cite{82}. However, in super-resolution microscopy this light also needs to be obtained within a limited timespan. Drift in the setup or the physical movement of a structure can blur a reconstructed image. Furthermore, approximately 10,000 - 100,000 single molecules need to be detected. These practical conditions set limits to the design of a super-resolution imaging technique. To detect sufficient photons from a single molecule, the angle under which light is collected through the objective needs to be as large as possible. An objective with a high Numerical Aperture (NA) is therefore a prerequisite for super-resolution microscopy (see figure 1.3A).
1.4.1 Inversion improves resolution

The fundamental difficulty with a high-NA objective is the short working distance. Since light under a large angle is observed, the distance at which objects are in focus is small, typically 100-170 µm. Many techniques that probe cell mechanics, however, involve microfabricated structures that make approaching the sample at such distances impossible. In these cases, imaging is either performed through this microfabricated structure or using a water-dipping objective. In both cases, it
is not possible to use a high-NA objective. Furthermore, devices that probe cell mechanics are typically made of Poly-Acrylamide (PA) or Poly(DiMethyl-)Siloxane (PDMS) that have a refractive index of 1.45 and 1.4, respectively [69, 83]. Imaging through structures with a different refractive index from glass (n=1.515) and water (n=1.333) further complicates high-resolution imaging.

Recently, we have been able to adapt techniques that either actively deform cells or probe cellular force exertion to enable the use of a high-NA objective. We adapted a substrate stretcher so the mechanical stretching was inverted and close to the coverslip (see figure 1.3B and D). The mechanical clamps are held in place ∼50 µm above a 100 µm coverslip, keeping the total distance from objective to cell and substrate within the desired WD. A thin PDMS sheet was used as a membrane of only 100 µm thickness, so the transverse strain would not shrink the sheet out of range of the WD. We have quantified the strain field of this device and performed successful initial experiments stretching live cells. Experiments with the inverted substrate stretcher are out of the scope of this thesis and will be discussed elsewhere.

In a similar inversion-approach, we adapted micropillars by adding flanking 50 µm spacers so we could invert them onto a 100 µm coverslip (see figure 1.3C and E). Cells remained viable on these inverted micropillar arrays and their mechanical behaviour seemed unaltered. This approach allowed us to measure cellular force exertion and perform superresolution microscopy simultaneously [19] (chapter 2). With the potential to probe cell mechanics and carry out high-resolution microscopy, we further focussed on fundamental cellular processes.

1.5 From mechanics to biology (and back)

The basic principle of a mechanical feature (e.g. deformation or stiffness) that influences a biological process has been termed mechanosensing (see figure 1.4). It is most clearly envisioned by the mechanical activation of a protein which induces a conformational change that leads to biological activity. This process can occur in several different ways, as will be described by means of individual examples. Mechanical activation can be caused by physical stretching, as was measured for p130Cas and talin [53, 57]. More well-studied are mechanosensitive channels, in particular in bacteria where the structure of the large-conductance mechanosensitive
channel MscL \[84\] for instance shows a large complex that opens when the membrane tension changes.

After mechanical activation, molecular adaptation is needed. This can be physical stretching (as is the case for p130Cas and talin) or opening of a pore complex (as is the case for mechanosensitive channels). Conformational changes of a mechanosensory protein or structure finally activate a biological pathway. This cascade of events can be performed through activation of a kinase, or by enabling a protein to bind to the mechanosensory protein and form a multi-protein complex (for p130Cas and talin, respectively). Mechanosensitive channels typically allow the influx of ions that in turn influence a wide range of biological processes. Kinase activation or protein binding in turn activates downstream biological signalling pathways. As many biological pathways are cascades with positive feedback, a mechanosensory mechanism can also further amplify the mechanosensory response through such a mechanism.

![Mechanosensing as a direct molecular mechanism](image)

**Figure 1.4**
Mechanosensing as a direct molecular mechanism. A mechanical activation causes a molecular adaptation. This can occur globally (cell-wide) through network adaptation or locally (at single- or several protein level) through direct molecular response. Finally a biological response changes functionality that feeds back onto the activation.

### 1.5.1 Mechanosensing through network reorganization

Manifestation of a biological phenotype is thus observed as a result of varying stiffness. However, the exact molecular mechanism that mediates mechanosensing is often not understood. The most striking mechanosensory observation was the stiffness-dependent differentiation of mesenchymal stem cells (MSCs) on poly-acrylamide gels \[9\]. MSCs showed a myosin-dependent response on substrates with a stiffness of 0.1 - 1 kPa,
10 kPa and 30-40 kPa with markers for neurogenic, myogenic and osteogenic differentiation, respectively. A follow-up experiment showed that it was not the local extracellular stiffness, but the global cell wide micron-scale stiffness that induced differentiation as confirmed by experiments on micropillars [10, 68]. More recently it was shown that the lamin-network around the cell nucleus adapted especially the laminA-content to changes in the extracellular stiffness [11]. Notably, a correlation between laminA:laminB ratio was found with tissue microelasticity in vivo. These observations imply that the ECM stiffness can cause a global cellular network adaptation as a mechanosensory response. A further fundamental understanding of how forces are transmitted throughout cellular networks is thus vital to understanding these mechanosensory processes.

Another striking global cellular mechanosensing response was termed mechanotaxis, in which cells migrate depending on mechanical cues. Mechanotaxis was observed by differential migration depending on shear flow [85]. Durotaxis is the most well-known subset of mechanotaxis that describes how cells migrate depending on the stiffness of their extracellular environment or substrate [86]. Again using micropillars, it was recently shown that durotaxis in fact depends on the global extracellular stiffness [17]. At a barrier where micropillars changed their diameter, the effective global stiffness greatly increases with micropillar diameter (see equation (1.2)). Indeed, at this barrier, fibroblasts migrated towards the stiffer micropillars, demonstrating the effect of another global mechanosensing mechanism. This phenomenon can be directly explained by the fact that fibroblasts [17, 19], epithelial cells [18, 87] and muscle cells [18, 88] exert pulling forces towards the cell body that increase with extracellular stiffness.

Changing the stiffness of the extracellular environment thus causes a fundamentally different response in cellular mechanics. Cellular mechanotransduction is mainly mediated by myosin-dependent contraction of the actin cytoskeleton [89]. To gain further insight into how forces are transmitted and shape the cell, we examined the effect of actin cytoskeletal orientation at the cellular periphery in relation to cell geometry and force exertion (chapter 3). Such insights in the force balance of cellular networks provide important cues into how cellular forces are transmitted globally.
1.5.2 Local activation through force

Another mechanism through which a cell can respond to extracellular mechanical cues is through direct local changes in molecular conformation. We and others have observed that cells in stiffer environments typically exert larger force \cite{17, 18} (chapter 4). Of particular interest in this respect is the focal adhesion (FA) complex \cite{90, 91}. A schematic of intracellular forces transmitted through the FA to the ECM is depicted in figure \ref{fig:1.5}. The cytoskeletal structure inside the cell comprises of intermediate filaments, microtubules and actin filaments. The actin cytoskeleton is connected to the FA, which comprises of a multitude of biologically functional interactions \cite{92}. Integrin heterodimers then span the cell membrane, connecting the FA complex to ECM proteins (e.g. fibronectin, collagen or laminin). One can imagine that mechanosensing through protein localization or -activation takes place in the FA, as forces are transmitted in this co-localization of mechanical stress and biological functionality.

The FA complex as a whole grows with local force exertion and its global assembly is force-dependent \cite{16, 19}. The reported size distribution of FAs does not increase accordingly with stiffness, so the force per protein increases with increasing stiffness. Mechanosensitive activity has already been indicated or reported for multiple FA proteins. An intriguing observation was made in the force-dependent unfolding of talin which enables binding of vinculin \cite{57}. Recently, single-molecule force spectroscopy and binding assays confirmed the unfolding of talin, and binding of vinculin upon forces of $\sim 5 \text{ pN}$, with a differential role for higher and lower forces \cite{93}. Forces in the range of 10 pN per protein are likely typical for the adhesion complex, as a minimum of 30-40 pN per integrin is the minimum force needed for cell spreading \cite{94}.

1.5.3 P130Cas as a mechanosensor

The mechanosensitive protein of our interest is the multi-functional scaffolding protein p130Cas \cite{95}. When fixed cell cytoskeletons were stretched on a substrate, it was observed that p130Cas would phosphorylate, indicating stretch-dependent signalling activation \cite{53}. The intrinsically disordered substrate domain \cite{96} has 15 YxxP motifs that can be tyrosine phosphorylated by Src. In cancer progression, it was shown that p130Cas promotes invasiveness in Src-transformed cells \cite{97}, giving this
1.5 From mechanics to biology (and back)

Figure 1.5
Local cellular force exertion. Zoomed in on an adhesion site, cellular contractile force (arrow) is exerted towards the cell body through myosin-motor activity (yellow) on the actin cytoskeleton and stress fibers (red). The actin cytoskeleton attaches to focal adhesion complexes (green) through many molecular interactions. Force is transmitted through integrin heterodimers (blue) that connect intracellular proteins to the extracellular matrix (pink).

biophysical process added relevance. In further studies it was shown that p130Cas directly influences actin polymerization and branching as well as FA dynamics [98, 99].

To further investigate the response of p130Cas in live cells and on varying stiffnesses, we performed experiments on Mouse Embryonic Fibroblasts (MEFs) lacking p130Cas (chapter 4). We compared MEFs with p130Cas-YFP reintroduced at endogenous levels to MEFs without p130Cas or without its two FA-targeting domains. We found that p130Cas does indeed have a mechanosensory function in a physiological stiffness range of 40-150 kPa, within the range of areolar tissue stiffness [42, 43]. Differential localization to FAs depends on the global extracellular stiffness. Furthermore, we found that this differential localization had an effect on the local force exertion characteristics on a single micropillar. Cells expressing truncated p130Cas (inhibiting localization to FAs) showed different force exertion characteristics, directly demonstrating the role of p130Cas in force exertion. P130Cas thus not only acts as a sensor, but also directly influences the mechanical response.

1.5.4 Force exertion during cell division

Finally, we investigated the cell mechanics implications of cell division. The process is evidently ubiquitous in life and development, and as such the mechanical progression is important to study. We characterized pro-
progression through the cell cycle (chapter 5) and found that the typical pulling force toward the cell center on a substrate was released. Instead, cells went through progressive steps in outward pushing forces that consistently preceded a well-known biological phenotype. We hypothesized a force balance that is set up by reorganizing the internal cytoskeleton and in particular the mitotic spindle and alignment of chromosomes.

A force-dependent role for kinetochore activation to continue through mitosis with kinase Aurora B was previously proposed [100]. We observed mitotic disturbance with a tripolar spindle coinciding directly with a lack of outward pushing and thus a disturbance of the force balance. These results show the need for mechanical integrity for progression through mitosis and direct proof of a mechanosensory effect in division.
1.6 Outline of this thesis

In chapter 2 our adaptation to micropillars that enables the use of high-resolution optical microscopy is presented. Simultaneous measurement of cellular force exertion on the ECM and high resolution live- and fixed cell microscopy shows normal cellular morphology and mechanics. Using direct STTochastic Optical Microscopy FAs are super-resolved and shown to be smaller structures than previously quantified. The connection between the intra- and extracellular matrix thus is shown to bear a high concentration of cellular forces.

In chapter 3 micropillar force measurements and fluorescence microscopy on live 3T3 fibroblasts show how extracellular force exertion correlates to the intracellular actin cytoskeleton. Locally, actin fibers co-orient with force exertion and globally the cellular shape shows circular arcs, described by a local mechanical equilibrium. The local orientation of the actin cytoskeleton influences curvature of arcs spanning the cell membrane and the guidance of force exertion.

In chapter 4 the response in function of p130Cas to variations in extracellular stiffness is described. Over an extracellular stiffness of poly-Acrylamide gels of 42-87 kPa, the presence of p130Cas changes FA formation. In fact, p130Cas localizes to FAs predominantly on micropillar arrays with a global stiffness larger than 47.2 kPa. Its differential localization also changes the force exertion dynamics on single micropillars, changing cell mechanics. P130Cas thus couples not just from mechanics to biological activity but also changes cellular forces.

In chapter 5 the cell division cycle is related to extracellular force exertion. For the first time, a passive extracellular force measurement of the evolution of a force balance throughout mitosis is quantified. Outward pushing forces from the cell center to the ECM increase throughout mitosis. The observed force balance proves vital in maintaining integrity of the mitotic spindle and succesful cell division.


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