

An explicit model to extract viscoelastic properties of cells from AFM force-indentation curves

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Atomic force microscopy (AFM) has become one of the most used techniques for quantifying the mechanical properties of soft materials such as living cells. AFM force-indentation curves are conventionally fitted with a Hertzian model to extract elastic properties. The elastic properties solely are, however, insufficient to describe the mechanical properties of cells. Hence, extending the quantification to assess, in addition, the viscous behavior, is necessary for a more adequate characterization of the mechanical properties.

Here, we expand the analysis capabilities to describe the viscoelastic behavior of the probed materials while using the same conventional AFM force-indentation curves. Our model gives an explicit relation of force and indentation and extracts physically meaningful cell mechanical parameters. We first validated the model on simulated force-indentation curves of a viscoelastic half-infinite space. Then, we applied the fitting model to the force-indentation curves of two hydrogels with different crosslinking mechanisms, polyacrylamide and agarose. We demonstrated that the viscoelastic properties extracted using our fitting model reflected adequately the distinct nature of these hydrogels. Finally, we characterized HeLa cells in two different cell cycle phases, interphase and mitosis and showed that mitotic cells have a higher apparent elasticity and a lower apparent viscosity when compared to interphase cells.

Our study provides a simple and rapid method, which can be directly integrated into the standard AFM framework, for extracting the viscoelastic properties of materials. This facilitates the exploration of advanced material properties in general and the understanding of complex biophysical processes in particular.

Studying mechanobiology of vascularised tumour and cancer cell intravasation using microfluidics

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Cancer metastasis remains as the most common cause of all cancer related – deaths. It is defined as the spread of cancer cells from their primary site of origin to a distant tissue. The primary tumour microenvironment is complex and composed of a myriad of cellular and acellular components. While the cellular components comprise of stromal, immune cells, lymphatic and blood vessels, the acellular components include the immensely complex extracellular matrix (ECM) composed of proteins, glycoproteins and proteoglycans. Both these components are interwoven and participate in tumour invasion through secretion of chemical factors as well as physical interactions between cell-cell and cell-ECM. One of the issues with understanding these mechanisms in depth is the need to mimic the complexity of such intricate interactions *in vitro*.

With the advancement of organ-on-a-chip technology, generation of human microvascular network *in vitro* has been possible. This has enabled studies of vascular and blood biology by opening horizons for modelling diseases such as tumour angiogenesis or thrombosis which serve as a starting point for regenerative medicines. Such platform offers the capability to capture biological complexity such as 3D spatio-temporal structure and functionality of the human microvasculature.

In this study, we have created a platform that replicates primary tumour growth and cancer cell escape into the nearest blood vessels in a process called intravasation. In order to do this, we take advantage of the versatility of microfluidic systems, where tiny, interconnected channels are etched into materials to achieve the desired features. By precise control of the materials, including cells, we are able to recapitulate the biological, chemical and mechanical cues of the human system. We employ cells of human origin which are embedded in a 3D supportive matrix similar to that of an *in vivo* tumour.

As a result, we observed that modulating the ECM components in the microfluidic chip leads to dramatic changes in the tumour and blood vessel interactions, causing vascularisation and intravasation events to happen. This highlights the importance of choosing a suitable ECM constituent to mimic the tumour microenvironment, which is often overlooked. This study is an ongoing effort to curtail the gap between *in vitro* 3D models and the *in vivo* scenario. Developing an understanding of the biophysical mechanisms underpinning tumour vascularisation and intravasation, in a physiologically relevant system is essential to identify potential barriers to prevent metastasis. This will advance our understanding of cancer behaviour and will facilitate therapeutic drug testing in the future.

Viscoelastic Properties of Ishikawa Spheroids Measured by pL-Ferrofluid Droplet Deformation Method

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The investigation of mechanical properties of tissues and cells stays as a hot topic for decades. Well-known methods, like nanoindentation or rheometer for local and bulk measurements, respectively, are often used for measuring viscoelastic material properties [1]. Determination of local mechanical properties in 3d can be done with manipulation of magnetic micro- or nanobeads [2, 3].

Here, we use a magnetic microrheometer setup that is based on the pL-ferrofluid droplet deformation method [4] in presence of a magnetic field for measuring viscoelastic properties of Ishikawa spheroids (Fig. a) [5]. In our setup, a Helmholtz arrangement of two electromagnetic coils generates a homogeneous magnetic field in between. The spheroid is placed in the center between the coils. When the lumen of spheroid is filled with ferrofluid (Fig b), a constant magnetic field is applied. According to the ferrofluid properties, the droplet elongates itself and stretch the spheroid (Fig. c) along the magnetic field lines. This time-depending elongation corresponds to the creep mode of viscoelastic behavior (Fig d), which can be described by a generalized Kelvin-Voigt model [6]. Values for viscosity and elasticity will be presented.

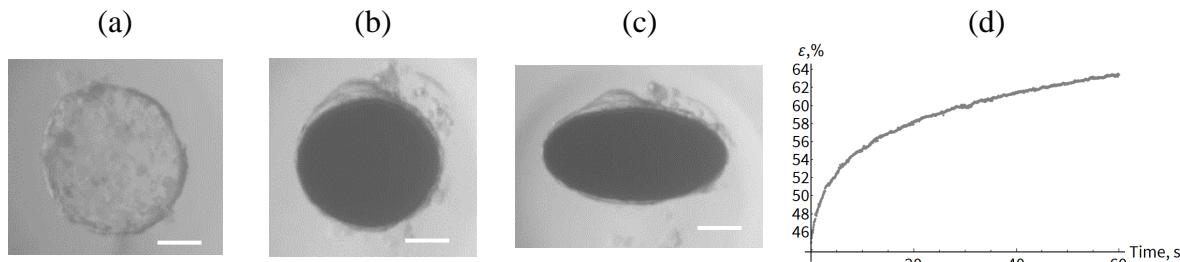


Fig. a) Ishikawa spheroid; b) Spheroid with ferrofluid in lumen; c) Elongated spheroid in magnetic field; d) Creep regime of spheroid under constant magnetic field. Scale bars on (a), (b) and (c): 50μm.

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Atomic force spectroscopy-based essay to evaluate oocyte post-ovulatory ageing

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Post-ovulatory ageing is a process occurring in the mature (MII) oocyte leading the unfertilized ones to apoptosis. The optimal time window of fertility for different mammalian species after oocytes maturation depends on its timeliness: the higher the time elapsed from the accomplishment of the MII stage, the lower are the chances of fertilization and development of a viable embryo. In the in vitro fertilization the selection of competent oocytes for intracytoplasmic sperm injection (ICSI) is mostly made by the visual inspection of the MII oocyte morphology, which does not allow to determine the oocyte post-ovulatory age. To evaluate it, specific tests can be performed but they involve some kind of staining which compromises the viability of the oocyte for reproductive purposes. Hence the need of a non-invasive analysis of oocyte ageing to improve the success rate of in vitro fertilization procedures.

Here we exploit atomic force microscopy to examine the evolution of the mechanical properties of mouse oocytes during in vitro post-ovulatory ageing. Different experimental setup were tested (Fig. 1A). Three hours before the occurrence of any visual morphological feature related to degradation, we observe a sudden change of the mechanical parameters: the elastic modulus doubles its initial value while the viscosity decreases significantly. These mechanical variations are temporally correlated with the release of the cortical granules as observed by fluorescence microscopy (Fig.1B).

These findings reveal that minimally invasive mechanical measurements are very sensitive to the status of the oocyte and they could be used as a label-free method to detect the post-ovulatory aging of oocytes in the in vitro fertilization procedures.

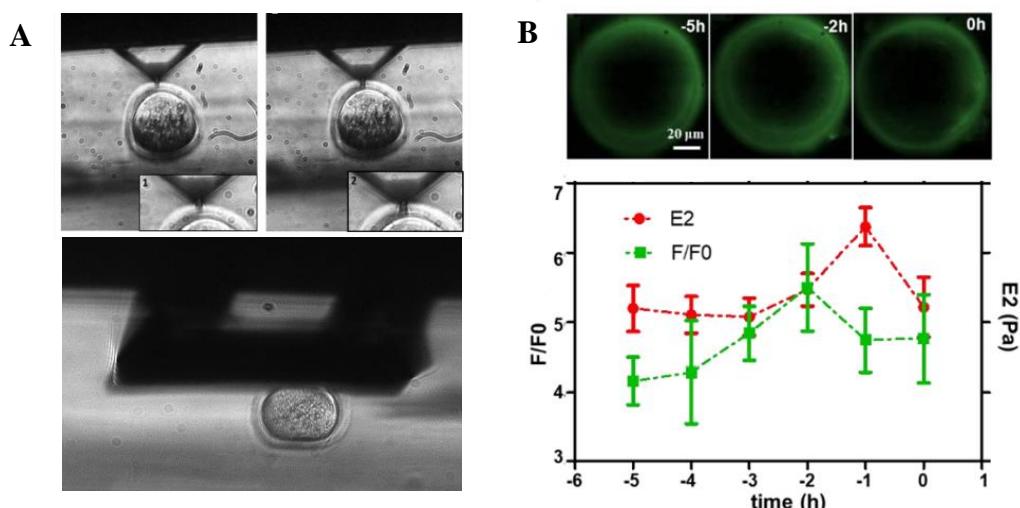


Fig.1: (A) Mechanical measurements of oocyte by AFM, indentation (upper panel) and stress-relaxation with macro-cantilever (lower panel). (B) An example of the fluorescence intensity variation related to CG exocytosis at three different times before the occurrence of the visual degradation (upper panel); trend of the FITC-LCA intensity signal (in green) and the second Young modulus (E2) variation of oocytes during in vitro post-ovulatory aging (in red) (N=25) (lower panel).

From Single-Protein to Single-Cell: a unique platform that combines optical tweezers and fluorescence microscopy for the study of Cytoskeletal Processes and Cell Mechanics

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Cells and protein filaments such as microtubules, protein droplets, actin and intermediate filaments are highly dynamic structures that constantly interact with each other and their surroundings. The forces governing these interactions play fundamental roles in essential biological process like cell division, motor transport, protein signalling or cell migration. Therefore, being able to study the mechano-chemical and mechano-biological pathways governing these interactions is essential to understand their nature better.

Force spectroscopy on a single-molecule and single-cell level permits exploring and manipulating these complex interactions. Our force-spectroscopy platform, which we commercialize as the C-Trap, integrates optical tweezers, fluorescence microscopy, and an advanced microfluidics system in a truly correlated manner. It enables live, simultaneous and correlative visualization and manipulation of molecular interactions for a wide range of forces (from picoNewtons to a nanoNewton) and with high temporal resolution (microseconds).

Here we present our experiments visualizing and quantifying the elastic properties of protein filaments, the motility of cytoskeletal molecular motors, the characterization of forces exerted by filopodia, or probe specific ligand-cell receptor interaction. These experiments show that the technological advances in hybrid single-molecule and single-cell methods can be turned into an easy-to-use and stable instrument that opens new venues in many research areas.

An *in vitro* model to study the effects of cyclic mechanical strain on human pulmonary epithelial cells in interaction with macrophages

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Rationale: During breathing, alveoli are constantly subjected to physiological mechanical stimuli. In lungs with acute respiratory distress syndrome (ARDS), a heterogeneous inflammatory lung disease, normal breathing is insufficient for gas exchange and mechanical ventilation has to be applied. In the inflamed lung, this results in unphysiologically high pressures and thereby mechanical stress, even when protective ventilation strategies are used. According to earlier experimental studies, mechanical strain increases pulmonary inflammation (biotrauma), but cannot induce inflammation in the healthy lung. With this background, we aim to investigate whether cyclic strain also inhibits the resolution of inflammation by influencing macrophage polarization (M1/M2) in the alveolus.

Methods: We optimized the culture conditions for the reproducible formation of closed monolayers of human alveolar epithelial cells (hAELVI cell line, InSCREENeX GmbH, Braunschweig, Germany) and primary human M1 and M2 macrophages. Therefore, hAELVI cells were grown for 1 - 14 days on polydimethylsiloxane (PDMS) silicone rubber with 5 - 50 kPa stiffness, resembling the stiffness of the healthy versus the inflamed lung. The elastomeric substrates are suitable for application of uniaxial cyclic strain (cell stretcher, IBI-2, FZ Jülich, Germany). To mimic pulmonary inflammation, hAELVI cells were stimulated with IFN-γ + TNF-α. Human peripheral blood monocytes (PBMC) were differentiated with GM-CSF or M-CSF and polarized with LPS or IL-4 + IL-10, respectively, to obtain M1 and M2 macrophages. Cell stretcher settings were chosen to resemble ventilation parameters (frequency = 0.3 Hz, 20% amplitude, for 1 - 24 hours). The effect of strain on the actin cytoskeleton, focal adhesions, adherens junctions and tight junctions was analysed by confocal fluorescence microscopy. Gene expression was quantified by qRT-PCR. M1 and M2 macrophages polarization was additionally analysed by flow cytometry.

Results: Stretcher experiments with hAELVI cell clusters after 24h were inconsistent. In contrast, after 14 days *in vitro*, the actin cytoskeleton, focal adhesions and adherens junctions reorganized fundamentally. Our data reveal the necessity to cultivate hAELVI cells for 14 days to obtain homogeneous and intact monolayered cuboidal cell cluster. Furthermore, hAELVI cells responded to inflammatory stimulation with increased gene expression of TNF-α, IL-6, TGF-β and IL-1β. Human PBMCs were successfully isolated, differentiated into macrophages and polarized into the M1 and M2 phenotype, as validated by comprehensive qRT-PCR and flow cytometry panels. Macrophage polarization did not change with substrate stiffness. Preliminary microscopic analyses of M1 or M2 macrophages indicated that macrophages, in mono-culture, do not respond to cyclic uniaxial stretch (1 *versus* 16h).

Conclusion: We successfully established a model suitable for characterization of cyclic mechanical strain in human alveolar epithelial cells and macrophages. We will combine these cell types in co-culture to study the interaction of macrophages and epithelial cells in the ventilated lung, with special focus on M1/M2 polarization.

Simulated microgravity modifications in musculoskeletal cells gene expression

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Gravity is one of the four fundamental forces that govern the universe and that we experience in daily life without realizing it.

The absence of mechano-stimulation caused by lack or reduced gravity (microgravity) has been widely studied since the beginning of the space age. This condition creates various cascade reactions in humans, resulting in a profound remodeling and adaptation of organs and tissues in the organism. In particular, osteopenia and sarcopenia are two of the first changes observed after space flights, which have a great impact on astronaut wellness.

We are studying the effects of culturing cells using a Random Positioning Machine (RPM), a tool capable of simulating microgravity. This instrument has a platform that moves on two rotation axes at a random speed and direction leading to the nullification, averaged over time, of the gravity force vector. We are studying two experimental murine models: the C2C12 and the MLO-Y4, which are respectively myoblast and osteocyte cell lines.

In the C2C12 cell line, we studied the expression of differentiation genes such as MyoD, MyoG, and the isoforms of myosin heavy chain (MHC) at different culture time points (1, 3, 5, and 7 days) both in-ground control conditions and on RPM. Through Real-time PCR analysis of early and late differentiation marker genes, we observed that RPM display a delay of the differentiation process that becomes statistically significant at 5 days in the case of the MHC 2 alpha mRNA expression ($P \leq 0.05$).

In the osteocyte cell line MLO-Y4, we noticed cell behavior changes after 5 days in RPM. In particular, the mRNA of connexin 43, a protein that forms gap junctions between cells with key functions in signal transduction, and in response to hormonal and mechanical stimuli, was downregulated in RPM cultures ($P \leq 0.0001$). Furthermore, the mRNAs of genes linked to cellular senescence such as P53 (tumor suppressor), P21, and P16 were found to be much reduced in simulated microgravity ($P_{53} = P \leq 0.01$, $P_{21} = P \leq 0.0001$). Finally, the quantification of the mRNA coding for BAX, a factor that regulates cell death, also showed a significantly reduced expression in RPM ($P \leq 0.001$).

Further experiments will be needed to fully understand the complex interplay that links the expression of these genes and microgravity exposure.

The MOlecular-Scale Biophysics Research Infrastructure (MOSBRI)

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The **MOlecular-Scale Biophysics Research Infrastructure (MOSBRI)** enables ambitious integrative studies of bio-systems at the crucial scale between atomic-resolution descriptions and cellular-scale observations. To reach its aim **MOSBRI** offers advanced integrative multi-technological approaches.

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Networking activities will increase the impact of **MOSBRI**, by disseminating knowledge through training events in Europe.

Advanced research projects will be launched to explore aspects underpinning the technologies and methodologies pipelines provided by **MOSBRI** and push them beyond their current limits. These projects will globally aim at improving performance, notably at the level of sensitivity, throughput, parallelisation, and capacity to extract information from the biological sample.

Photoelastic Gel Microscopy

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Cells continuously exert and sense forces through adhesion complexes bridging the cytoskeleton and the extracellular matrix. This force exchange modulates important physiological processes including stem cell differentiation, cell migration and tissue morphogenesis; as well as pathological processes such as cancer progression¹. Measuring cellular forces is therefore at the basis of deepening our understanding of *mechanobiology* and translating this knowledge into useful diagnostic tools.

The most common approach to measure cellular forces exerted at the cell-matrix interface *in-vitro* is 2D traction force microscopy (TFM)^{1,2}. TFM is based on measuring the in-plane deformation of an elastic gel (e.g., polyacrylamide) in response to cellular forces by optically measuring the displacement of fiducial fluorescent markers embedded in the gel. Knowledge of the initial position of the markers allows for the quantification of a displacement map, and for the stress field to be computationally reconstructed. Despite being the gold standard method to measure cellular forces, TFM is an experimentally complex technique, and force reconstruction is a mathematically *ill-posed* problem; which means that a small amount of noise in the displacement field generates a large error in the computed force field¹. These factors have prevented the widespread use of TFM in non-specialized labs, and ultimately its translation as a useful diagnostic tool².

Here, we propose a method to measure cellular forces by monitoring the stress-induced birefringence of mechanically tunable gels. The measured intensity under stress is a function of the retardation (R), which is proportional to the in-plane maximum shear stress. Because R is proportional to stress (force/area), if a very small force is applied over an equally small area, the sensitivity of the approach is theoretically infinite (besides the constraints introduced by the optical system). Our preliminary data highlights the suitability of the method to measure cellular forces in a label-free and direct manner.

We envisage that our method, called *photoelastic gel microscopy*, will offer the possibility to measure cellular contractile forces in non-specialized labs, and eventually be used as a low-cost diagnostic tool for mechanopathologies.

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Nanomechanical sensors in partial wetting condition for investigating local cell motion during adhesion process

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Cell adhesions and cell migration are inter-dependent processes whose investigation is of great interest in human development, inflammation and disease pathogenesis. Recent studies have shown that commercial cantilevers can be used as nanomotion sensors that are able to convert the small movements of living cells into dynamic oscillations [1].

In these studies the cantilevers were totally immersed in liquid environment, which affects system sensitivity by damping in liquid medium due to viscosity, spurious deflection by medium refraction or by scattering from floating debris. To overcome this limitation and improve the performance of nanomotion sensors, we propose a partial-wetting nanomotion detector (PWMD) operating in a partial wetting condition. Indeed, this configuration enhances the quality factor of the cantilever, by reducing liquid-sensor interface [2].

Our nanomotion sensor consists of a silicon chip including a 500 µm large window where rectangular silicon nitride cantilevers are carved in the suspended membrane and separated from the latter by a groove. To evaluate the effects of geometrical features of these sensor we designed cantilevers having different length, width and groove width.

We applied the PWMD to the investigation of nanomotion of breast cancer cells (MDA-MB-231) in the adhesion process. To perform partial wetting measurements we designed and fabricated a special chamber of measurements that allowed cells to adhere on one side of the cantilever which was functionalized with Aptes (3-aminopropyltriethoxysilane), while keeping dry its backside where laser beam is reflected. The cantilever deflection was then detected by using the cantilever detection system of a JPK Nanowizard II AFM, mounted on top of an inverted microscope.

We observed that longer cantilevers show a better sensitivity to cell nanomotion, hence cantilevers with 200 µm length, 30 µm width and a groove width of 2 µm were selected for cell experiments. By using these cantilevers we observed an increase of cantilever deflections when living cells adhere and moves on the cantilever or across the groove. Simultaneously to deflection measurements, we acquired a movie to correlate the cantilever deflection with the cell motion. After treatment with cytochalasin D, the cantilever deflection significantly decreases along with the cell motion.

We demonstrated that the PWND represents a promising tool to analyze in real time cell adhesion dynamic and the effect of drug interfering with this cellular process.

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A novel, organotypic, bi-compartmental platform towards replicating tissue barriers

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Introduction

Microphysiological systems (MPS) are a novel methodology for pathophysiology studies with a higher predictive power to the respect of conventional cell culture devices [1]. We have developed TToP, a novel MPS, that allows compartmentalized cell/tissue cultures enabling to monitor cell growth in real time and to easily recover and reuse the biological material, maintaining the morphological structure and vitality. A biological validation has been carried out in two case studies: i) Caco-2, as a model of intestinal epithelial barrier and ii) EAhy-926 as a model of vascular endothelial barrier, evaluating confluence and differentiation.

Methods

CO₂ Laser cutting rapid prototyping technology [2] has been applied to realize a 12-well plate compatible static system (Figure 1A). Polycarbonate 3 µm (EAhy-926) and 5 µm (CACO-2) pore size membranes have been hosted in the system. Growth has been monitored by Acridine Orange (AO) stain of living cells. Confluent cells were then fixed in paraformaldehyde, stained by DAPI (nuclei) and lineage-specific antibodies (EAhy-926: CD-31, VE-Cadherin; CACO-2: HEA, JAM) and extracted from the system by means of a controlled retrieval procedure.

Results

In the first set of experiments (Figure 1 B, C) Caco-2 confluent monolayer polarization was observed by confocal microscopy, after the retrieval of the membrane from the system, as presence of expressed JAM at the apical side and of HEA at the basal side, respectively.

In the second set of experiments (Figure 1 D, E) EAhy-926 cells phenotype (CD31, VE-CAD), and confluence (DAPI) were observed in all samples. Sample extraction procedure allowed to maintain, in all cases, morphological structure and vitality.

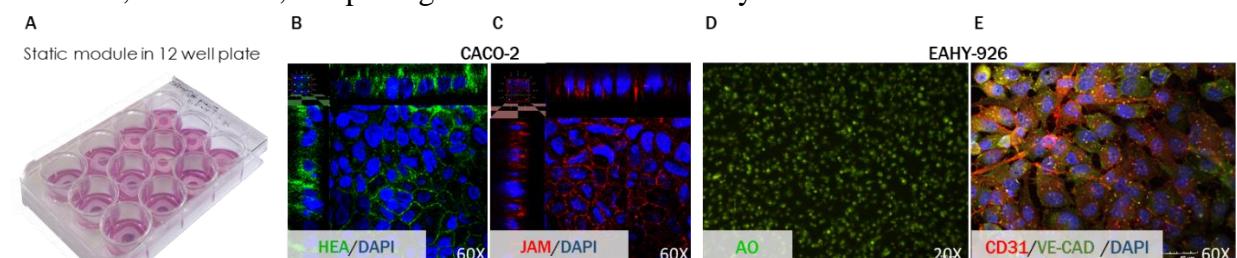


Figure 1: A) Static modules inserted in a commercial 12-well plate. B-E) Representative live fluorescence images of EAhy-926 and CACO-2 cells cultured in TToP static module.

Conclusions

TToP static module ensured simple and standard compartmentalized culture condition, allowing to obtain confluent, differentiated, and polarized monolayers with high reproducibility. Moreover, the novel features allowed to extract the biological sample in a controlled way, to perform imaging analysis. In perspective, we will design a compatible dynamic module to subject cells to perfusion and chemical gradients, both in single and co-culture conditions.

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Unravelling the mechanotransduction driving nanokicking-induced osteogenesis with a new single-dish nanokicking device

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The nanokicking technology, developed at the University of Glasgow and the Strathclyde University, aims to induce osteogenesis of Mesenchymal Stem Cells (MSCs) by applying vertical vibrations in the nanoscale (Nikukar et al., 2013). Research has suggested how this approach, under optimal parameters including frequency and amplitude of the vibrations, significantly increases osteogenesis compared to conventional biochemical methods.

Currently, these nanovibrations are driven by bioreactors that can accommodate two multiwell plates and allow for the application of this stimuli during weeks, either in 2D or in 3D. This has allowed for the assessment of, not only osteogenesis markers expression and mineral deposition analysis, but also for the study of mechanosensitive channels. Namely, Piezo1 channel expression has been suggested to significantly change in the different differentiation stages induced by the nanovibrations, as well as pERK levels are decreased when using a Piezo1 inhibitor compared to the control conditions. This leads us to propose that Piezo1 could play a fundamental role in the mechanotransduction of nanokicking.

As previously stated, the currently used bioreactor has been developed under the aim of long-term assessments. However, for the study of mechanotransduction, real-time analysis is needed. Moreover, considering that Piezo1 is a calcium channel that activates within milliseconds after its stimulation, this new bioreactor would need to meet different geometry and dimensional requirements in order to allow for its integration into a microscope for real-time analysis.

Under this aim, a new single-dish nanokicking device has been designed and mounted, with the help of Elbatech, in which the nanovibrations are driven by a piezo actuator in the shape of a ring, placed between a petri dish and a 3D printed holder, in which nanovibrations are driven by a custom made amplifier wired to the piezo ring. Soon, assessment of MSC osteogenesis using this bioreactor will be done as a proof of concept in order to prove whether this new bioreactor functions in the same manner as the currently used, which will be followed by the study of Piezo1 activation in real time when the vibrations are applied by analysing cellular calcium influx in real time when the nanovibrations are applied. This could later be extended to the study of different mechanosensitive channels and cellular components to assess their involvement in nanokicking mechanotransduction.

ECM-resembling Viscoelastic Hydrogels for Tissue Engineering Applications

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Introduction: It is known that biological tissues and the extracellular matrix (ECM) *in vivo* are not linearly elastic but exhibit a complex mechanical behavior in response to mechanical loading, such as stress relaxation and creep⁽¹⁾. However, only recently have there been efforts made to understand the role of ECM viscoelasticity in regulating cell behavior using 3D tissue-model hydrogels⁽²⁾. Results suggest that viscoelasticity affects spreading⁽³⁾, proliferation⁽³⁾ and differentiation⁽⁴⁾ of stem cells, in ways not anticipated from our previous understanding of mechanobiology which was largely based on results obtained on 2D elastic hydrogels such as polyacrylamide. Thus, the role of time-dependent mechanics on cell biology remains largely unclear, and ripe for further exploration. Here, we propose a novel approach to develop 3D ECM-resembling viscoelastic hydrogels by tuning their loss modulus independently of their elastic modulus.

Experimental methods: Polyethylene glycol (PEG)-Acrylate (PEG-AC) and PEG-Maleimide (PEG-MAL) gels were prepared using photopolymerization and Michael-type addition reaction, respectively. Viscoelastic hydrogels were synthesized by altering the branching and molecular weight (MW) of the macromers, while also varying the MW of the crosslinker, PEG-dithiol (SH-PEG-HS). The ratio of crosslinking molecules was kept constant (AC/MAL: SH, 1:1). The bulk mechanical properties of the hydrogels were measured using bulk rheology.

Results and Discussion: 8 arm and 4 arm PEG-AC and PEG-MAL of different MWs (20kDa and 10kDa) were combined with PEG-SH of different MWs (2kDa, 5kDa, 10kDa) to create synthetic viscoelastic hydrogels. We observed that through different combinations of macromer and crosslinker we could create groups of hydrogels with similar shear elastic modulus (set 1 with G' of ~400 Pa and set 2 with G' of ~170Pa), but different loss modulus (set 1 with G'' ranging from ~20-60Pa and set 2 with G'' ranging from ~5-25Pa). Specifically, 8arm 20kDa PEG-Ac hydrogels crosslinked with 10kDa PEG-dithiol showed the highest viscosity, with a $\tan(\delta)$ of 0.2.

Conclusions: We have developed a system through which we can modulate viscoelasticity of synthetic PEG-based hydrogels which can be functionalized with full-length ECM proteins to resemble the native ECM environment. Specifically, hydrogels with similar elastic modulus but tuneable loss modulus are created by altering the structure of the macromer and crosslinker. As tissues within our body are viscoelastic, creating matrices that more closely match native tissue-mechanics may substantially advance our understanding of cell-matrix interactions during physiology and disease.

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Exchange kinetics of desmosomal transmembrane proteins under the influence of mechanical stress

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The main function of desmosomes is to connect cells for the transmission of forces in epithelial tissues. Because mechanical loads are naturally changing with time, desmosomes are dynamic structures that assemble and disassemble to adapt to the momentary status. The desmosomal transmembrane proteins desmocollin and desmoglein mediate adhesion between adjacent cells, but little is known about their behaviour upon mechanical stress.

We studied monolayers of Madin-Darby Canine Kidney (MDCK) cells that are stably transfected with a GFP-tagged desmocollin 2a (Dsc2a). Cells were cultivated on silicone elastomer chambers and desmosome maturation induced by a calcium switch. A mechanical load was applied to these monolayers under live cell conditions by application of uniaxial, cyclic stretch. Afterwards, protein exchange dynamics were analyzed by Fluorescence Recovery After Photobleaching (FRAP).

We found that recovery curves could be fitted by a single exponential. This indicates that exchange kinetics and not diffusion was the rate-limiting step. Without mechanical stretching, Dsc2a is a stable component of desmosomes with a mobile fraction of 23% and a t_{halfmax} of 61 seconds. In order to assess whether desmosome maturation influences the exchange kinetics of Dsc2a, we analyzed different durations of high calcium incubation. Young desmosomes after three days incubation were compared to mature desmosomes after 13 days. We found that desmosome maturation seems to influence Dsc2a exchange kinetics by decreasing the mobile fraction. Subsequently, we probed the exchange kinetics of Dsc2a directly after subjecting the cell monolayer to 2 hours of cyclic stretch. First results indicate that mechanical stretch enhances Dsc2a exchange kinetics. Interestingly, Dsc2a exchange kinetics seem to remain increased even 24 hours after mechanical stretch. In summary, our preliminary results point towards a subtle, but long-lasting effect of mechanical stress on Dsc2a exchange kinetics.

Mechanical properties as identifier for viral conformational changes

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Infection by human papillomavirus (HPV) is the cause of the majority of cervical cancers. The attachment of the virion to the primary cell receptors, heparan sulfate proteoglycans (HSPG), initiates HPV infection cycle. HSPG binding triggers conformational changes in the viral capsid, and here we tested its effect on the viral mechanical properties. By applying Atomic Force Microscopy nanoindentation, we studied HPV after incubating with four putative HSPG mimics, respectively. In particular, the mimics, including heparin, are composed of saccharide backbones with different lengths. It turned out that the shorter length mimics did not affect the mechanical properties of the virus. However, like heparin, the mimics composed by the longer saccharide backbones could effectively trigger the switch in viral mechanics. The mechanism of longer length mimics inducing the conformational changes was revealed by comparing the mechanical properties of wild-type HPV with a mutant type upon heparin binding. Moreover, we found the heparin binding induced changes in the mechanical properties are reversible upon removal of heparin. Hereby we provided a first glimpse of how the mechanical properties of HPV are changed after HSPG mimics binding. Our results will help building a clear picture of the mechanism of HPV infection and are beneficial to optimize the design of anti-HPV medicines that specifically target blocking HPV infection at its first step.

Rheology of rounded mammalian cells over continuous high-frequencies

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To better link viscoelastic properties of living cells with molecular events we introduce rheological measurements of rounded cells. Performed in the range of 1-40 kHz such experiment such assay elucidates the link between cellular and molecular properties, such as polymer relaxation and monomer reaction kinetics [1]. The assay uses a photothermally actuated microcantilever to confine a single, rounded cell on a second microcantilever (see Figure 1), which measures the cell mechanical response across a continuous high-frequency frequency range. Cell mass measurements [2] and optical microscopy are co-implemented. The fast high-frequency measurements are applied to rheologically monitor cellular stiffening. We find that the rheology of rounded HeLa cells obeys a cytoskeleton-dependent power-law, similar to spread cells. Cell size and viscoelasticity are uncorrelated, which contrasts an assumption based on the Laplace law. Together with the presented theory of mechanical de-embedding, which abolishes the condition to stay away from instrumental resonances we present ways to simplify our approach and make it applicable to setups with just a single cantilever.

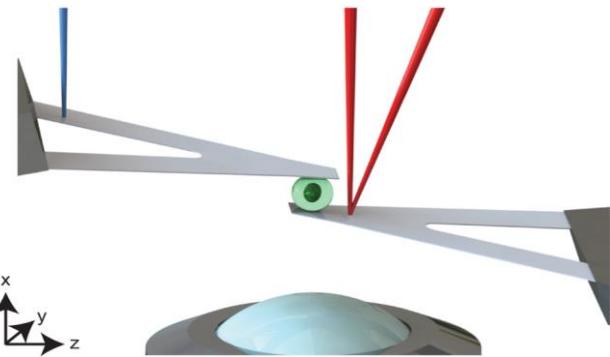


Figure 1 Experimental Setup. Two opposing cantilevers confine a cell in a parallel plate setup to perform rheological measurements of a single cell.

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The role of PADI2 on oligodendrocyte differentiation enhanced by mechanotransduction

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Oligodendrocyte precursor cells (OPCs) are mechanosensitive cells, since their survival, proliferation, migration and differentiation are influenced by mechanical cues. Our group demonstrated that substrates mechanically compliant with the rat brain (6.5kPa) enhanced the differentiation of OPCs *in vitro* and this effect was potentiated in presence of laminin-2, an extracellular protein that promotes oligodendrocyte (OL) differentiation. Recently, using magnetic resonance elastography — a non-invasive imaging technique to assess the rheological properties of tissues —, it was shown that brain softening occurs with physiological ageing. Strikingly, individuals with multiple sclerosis (MS) present exacerbated softening when compared with age and gender-matched controls, presumably due to deregulated extracellular matrix, cell death and consequent loss of mechanostasis (mechanical homeostasis). Taken together, data suggest that changes in mechanical properties of the brain might have an important impact on cellular processes of OLs (namely their differentiation), with specific relevance in the context of MS.

The mechanotransduction mechanisms involved in OL differentiation have not yet been fully elucidated, but it was reported that mechanical forces induce changes in chromatin structure, namely through mechanisms dependent on histones methylation. Gene expression and cell fate are controlled by epigenetic mechanisms, which play a key role on OL biology. Hence, we are dissecting the mechanisms underlying the enhancement of OL differentiation by mechanotransduction focusing on epigenetic modifications, namely citrullination by peptidyl-arginine deiminase 2 (PADI2).

We observed changes on epigenetic markers through mechanisms dependent on histones citrullination and PADI2 protein level in OLs differentiated on substrates with distinct degrees of stiffness, suggesting a novel chromatin regulation mechanism on OLs in response to mechanical cues. Moreover, in the absence of PADI2, MBP expression decreased, hampering OL differentiation. We hypothesize that PADI2 acts through cytoskeleton changes, since PADI2 is a regulator of RhoA and Rac1, well known modulators of actin and myosin. Indeed, PADI2 inhibition leads to a decrease of RhoA during OL differentiation.

Furthermore, we assessed RhoA activity by evaluating the phosphorylation level of cofilin-1, a proximal downstream target of the kinase cascade triggered by this GTPase. We observed a lower phosphorylation of cofilin-1 upon PADI2 inhibition, indicating reduced RhoA activity. Here, we dissect a possible mechanism of OL differentiation modulation by mechanotransduction. These results emphasize the significance of the work in the context of MS, and the importance of mimicking extracellular stiffness for OL differentiation in culture.

Biased contact guidance results in directed collective cell migration

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Ratchetaxis, the phenomenon by which cell migration can be guided by a repeated local anisotropic cue in the cell environment, has been observed for isolated single cells in several experiments. However, this effect has been little studied in dense cell populations.

Here, we study the behavior of bronchial epithelial cells (HBECs) plated on factory roof-like asymmetric substrates. Although isolated single cells are not influenced by the asymmetry of the pattern, interacting yet sub-confluent cells are highly sensitive to it, resulting in unidirectional migration. The direction of migration itself is non-trivial and depends on the substrate's geometrical properties (height, depth and ratchet asymmetry).

These preliminary results point towards a new mechanism of directed migration that depends on cell density. We propose that such a mechanism could be relevant *in vivo* in development and might be put to use in cell-sorting devices.

Micropatterning and metal-induced energy transfer show the effect of tension on the height of the actin cytoskeleton

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Cells adapt their actin cytoskeletons architecture to structural cues of the environment in all three dimensions. How shape influences the actin architecture in the imaging plane is well studied. Nevertheless, how manipulating cell shape influences the actin cytoskeletons organization normal to the imaging plane is unstudied, but crucial for an understanding of the mutual influence of cell shape, cell tension and actin architecture.

Metal-induced energy transfer (MIET) is a super-resolution microscopy technique perfectly suited for studying the organization of the actin cytoskeleton in this dimension of the actin cytoskeleton in response to different stimuli [1,2].

To study the effect of shape on the z-dimension of the actin cytoskeleton we combine MIET with protein micropatterning on gold [3]. This allows us not only to precisely manipulate the shape of the cell but also to regulate forces by changing the shape while studying specific actin structures with super-resolution in the third dimension.

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New mechanical activation mechanism of cell-surface metalloproteinases through a mechanosensitive ion channel

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Both epithelial and endothelial cells are constantly exposed to mechanical forces, such as the stretching of cells during respiration or, in the case of endothelial cells, the shear stress caused by the blood flow. These mechanical stimuli are important for the functional maintenance, such as structural integrity and a proper inflammatory response and wound healing. Thus, the proliferative and migratory potential of cells, permeability and the expression profile of inflammatory markers are affected. However, a chronic pathological alteration of these mechanical stimuli can also contribute to the development or progression of diseases. Mechanical stimuli are sensed, among others, by mechanosensitive (MS) ion channels which rapidly convert the stimuli into an intracellular signal and, thus, influence the cellular functionality mentioned before. With their limited proteolytic cleavage of chemokines, adhesion molecules, receptors, growth factors, and signalling molecules, cell-surface metalloproteinases can play an important role to immediately react with a functional response of the cell to the mechanical signal. In this study we show a new activation mechanism for certain members of the metalloproteinase family via the activation of a MS ion channel by chemical activation and by activation with mechanical stimuli, such as stretching or shear stress. The increased activity of selected metalloproteinases after the specific stimulation of a MS ion channel was measured via protease-specific shedding activity assays and ELISAs. The specific signal transduction via this MS ion channel was validated via lentiviral shRNA-mediated knockdown. The increased shedding activity in response to stimuli of the MS ion channel was strongly reduced in the knockdown cells, which further underlines our findings. Additionally, specific inhibitors were used to investigate the involvement of known signaling molecules in the activation of metalloproteinases. Finally, first results indicate a relevance in several physiological processes such as endothelial and epithelial cell proliferation and migration. In summary we could show that distinct metalloproteinases can be activated by mechanical stimuli mediated by MS ion channels both in epithelial and endothelial cells. Due to the versatile nature of the investigated metalloproteinases, these findings should be important for the understanding of many physiological and pathophysiological processes in endothelial and epithelial cells, such as tissue development, maintenance of tissue functions, inflammatory response, fibrosis and cancer.

How to get in shape - Multiscale Mechanics of Cell Sheet Folding

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Understanding how cellular changes and tissue folding drive morphogenesis during embryogenesis is one of the biggest challenges of developmental biology. We are aiming to gain a mechano-chemical understanding of morphogenetic processes in three dimensions. Gastrulation is one of the most crucial steps in animal development. Invagination is the evolutionary basic form of gastrulation, occurring in cnidarians, echinoderms and cephalochordates, and plays an important role metazoan organogenesis (e.g. eye cup formation). The spherical embryos of the basic chordate *Amphioxus* and the microalga *Volvox* [1-3] consist of a cellular monolayer and allow comparative studies of invagination across kingdoms. We are combining biophysical measurements and computational tools that enabled quantitative studies on cell sheet folding to reveal mechanical tissue properties and their function during gastrulation. A combination of advanced imaging and computational modelling is used to correlate cell shape changes, tissue contractility and the occurring tissue deformations. The associated internal stresses are determined through laser ablation experiments which allow conclusions on the underlying forces as well as the elastic properties of dynamic cell sheets. We show that local tissue bending has to be complemented by expansion and/or contraction in adjacent areas stressing the importance of global mechanics.

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Coupling of cell mechanoresponse with cell division rate in model epithelium

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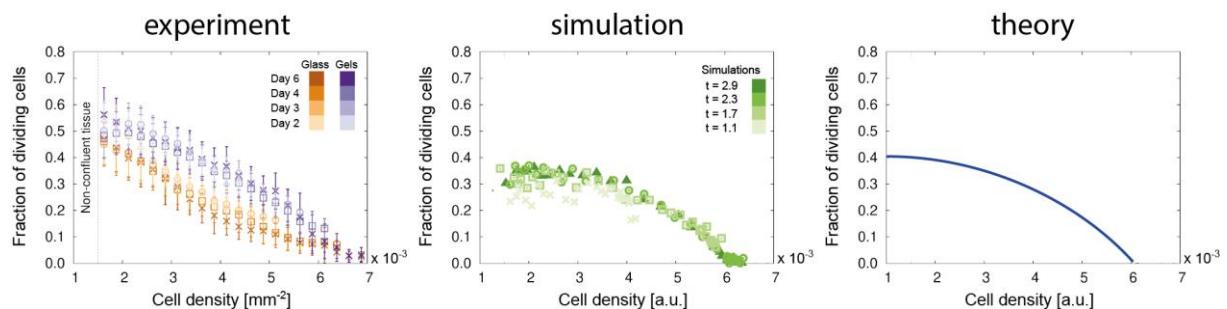
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The extracellular microenvironment (ECM) of epithelial cells is known to mechanically govern the properties and behavior of cells and tissues. Among others, the stiffness of the ECM has been proven to affect cell differentiation, as well as its average size and motility. On the contrary, the effect of ECM stiffness on the division rate of cells in tissues has not been analyzed in detail to our knowledge. In this work, we use MDCK-II cells grown on glass and 11 kPa PDMS substrates to provide evidence for a division rate that depends only on the local cell density, in ECM stiffness dependent manner. Interestingly however, the proliferation of cells was found independent of age of the model-tissue, its internal structure or state.

Based on these results, we provide a theoretical model for tissue growth in a local microenvironment with well-defined average cell-density, able to predict characteristic aspects of the density-dependent proliferation curves. The mechanical consideration of the dependence of the division rate on the cell size is elucidated by comparing the model with Dissipative Particle Dynamics (DPD) tissue simulations, which capture well the experiments. Consequently, we establish a procedure for the extraction of parameters associated with the model from either simulation or experimental data. This work therefore sheds a new light on the influence of the ECM stiffness on the maturation of epithelial tissues.



Experimental evidence for dependence of the proliferation rate on both local density as well as substrate stiffness with accompanying simulation data and theoretical model predictions supporting this result.

Force sensing on cells and tissues by atomic force microscopy

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Biosensors are aimed to detect tiny physical and chemical stimuli in biological systems. Physical forces are ubiquitous, being implied in all cellular processes, including cell adhesion, migration, and differentiation. Given the strong interplay between cells and their microenvironment, the extracellular matrix (ECM), the structural and mechanical properties of the ECM play an important role in the transmission of external stimuli to single cells within the tissue. Vice versa, also cells themselves use self-generated forces to probe the biophysical properties of the ECM.

ECM mechanics influences cells’ fate, regulates tissue development and show peculiar features in health and disease conditions of living organisms. Force sensing in biological systems is therefore crucial to dissect and understand complex biological processes, such as mechanotransduction.

Atomic Force Microscopy (AFM), which can both sense and apply forces at the nanoscale, with sub-nanoNewton sensitivity, represents an enabling technology and a crucial experimental tool in biophysics and mechanobiology.

In this work, we report on the application of AFM to study of biomechanical fingerprints of different components of biological systems, such as the ECM, the whole cell, and cellular components, like the nucleus and the glycocalyx. We show that physical observables like the (spatially resolved) Young’s modulus of elasticity of ECMs or cells, and the effective thickness and stiffness of the glycocalyx, can be quantitatively characterised by AFM. Their modification can be correlated to changes of the microenvironment, physio-pathological conditions, or gene regulation.

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Relation between tissue homeostasis and mechanosensitivity in model epithelium

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Despite recent efforts to understand homeostasis in epithelial tissues, there are many unknowns surrounding this cooperative steady state. In the context of cell morphology, single cell studies set mechanosensitivity as an important regulatory process. However, mechanoresponse in tissues remains heavily debated. Here we show using MDCK-II cells that changes in matrix stiffness induce a non-equilibrium transition from tubular to squamous epithelial tissues. Despite adopting different cell shapes and densities, all homeostatic states display equivalent topologies. This suggests that the latter property is actively targeted in homeostasis. On the contrary, we observe a dramatic change in the self-assembled organization of the colonies on the macroscopic scale. Such behavior is recovered in simulations by introducing stiffness-dependent activity. Our results unequivocally relate the mechanosensitive properties of individual cells to the evolving macroscopic structures, an effect that could be important for understanding the emergent pathology of living tissues.

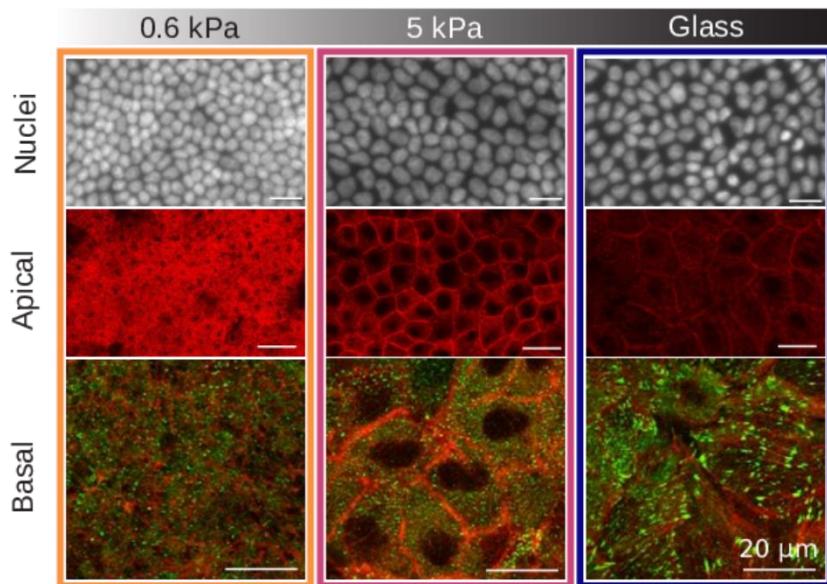


Figure 1: Nuclei, apical actin, and basal actin (red) along with paxillin staining (green) for MDCK-II tissues at homeostasis, grown on substrates of various stiffness, as highlighted above the picture.

TOWARDS HIGH THROUGHPUT CELL MECHANOSENSITIVITY ASSAYS

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Introduction

Cell mechanosensitivity is the ability of cells to sense mechanical stimuli arising from their surrounding environment or from within the body [1]. It is inevitably followed by mechanotransduction, the translation of the mechanical stimulus into biochemical signals [2]. Both processes play a crucial role in cells' fate and allow them to adapt to their physical surroundings by remodeling their cytoskeleton, activating different signaling pathways and changing their gene expression, ultimately controlling physiological processes such as proliferation, differentiation, migration, and apoptosis [3].

Dysregulation of cell mechanosensitivity results in cell dysfunctions and eventually various pathologies, such as cardiovascular diseases [4], osteoporosis [5], intestinal problems [6]. Currently, cellular mechanosensitivity is assessed mainly using single cell biophysical methods. While this approach allows us to characterise the mechanical response of single cells with very high resolution, it offers a very limited throughput. The measure of mechanosensitivity has the expectation to provide physiologically-relevant information, but a paradigm change is required to translate this concept towards biomedical applications, moving to high throughput single cell analysis.

Materials and Methods

Here we propose a concept design of a high throughput mechanosensitivity assay based on the integration of microfluidic and fluorescent microscopy. Floating cells will be aligned within the microfluidic channel and mechanically stimulated while passing through a neck in the channel. In order to track cells' response, we will use fluorescent ion sensitive dyes and a fluorescent inverted microscope. Studies on cell mechanosensitivity could contribute to the discovery of novel therapeutic strategies for treating diseases with mechanobiological elements.

Acknowledgements

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Application of Large Area Mapping AFM for Automated Structural and Mechanical Analysis of Developing Cells and Tissues

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Active forces in biological systems define the interactions between single molecules, growing cells and developing tissues. Further development of novel biomaterials for tissue engineering is driven by the biomechanical and molecular cues provided to cells by their environment which are crucial parameters that influence motility, behavior, and the fate of progenitor cells.

AFM can be successfully applied for comprehensive nano-mechanical characterization of single molecules, cells and tissues, under near physiological conditions. Currently, the trend is to extend this by studying the mechanobiology of living cells while evaluating their structure and the interaction with their cell culture substrates. In particular, it is interesting to understand how cell behavior is driven by the cytoskeletal dynamics and cell mechanics in typical cell culture scaffold scenarios. We will introduce the concept of automated large area multiparametric characterization of densely packed cell layers and highly corrugated tissue samples, where full automation, smart mechanical sample analysis, multiple scanner technology, and optical integration is critical for data throughput and reliable correlative microscopy. We will discuss how these developments, in combination with advanced optical microscopy techniques, can overcome the inherent drawbacks of traditional AFM systems for characterizing challenging biological samples.

Cells adapt their shape and react to the surrounding environment by a dynamic reorganization of the F-actin cytoskeleton. We will demonstrate how cell spreading and migration in living KPG-7 fibroblasts and CHO cells, can be studied with high-speed AFM and associated with spatially resolved cytoskeletal reorganization events. We will further extend this with high-speed mechanical mapping of confluent cell layers, which in combination with optical tiling can be applied to automated analysis of large sample areas.

External mechanical stress is known to influence cell mechanics in correlation to the differences in actin cytoskeleton dynamics. As a tool for analyzing the complex cellular mechanobiology, we went beyond purely elastic models, and performed sine oscillations (up to 1 kHz, amplitude 5-60 nm) in Z while in contact with the surface to probe the frequency-dependent response of living fibroblasts. We will further discuss how to calculate the viscoelastic properties, characterized by the dynamic storage and loss modulus (E' , E'') distribution in such samples.

In the past, investigating large and rough samples such as tissues and hydrogels using AFM was challenging due to the limited z-axis of the AFM. Using osteoarthritic cartilage as an example, we will demonstrate how a newly developed hybrid of a motorized and piezo stage enables multi-region AFM probing over a large, rough sample area while providing additional correlative optical data sets.

StemBond hydrogels control the mechanical microenvironment for pluripotent stem cells

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Studies of mechanical signalling are typically performed by comparing cells cultured on soft and stiff hydrogel-based substrates. However, it is challenging to independently and robustly control both substrate stiffness and extracellular matrix tethering to substrates, making matrix tethering a potentially confounding variable in mechanical signalling investigations. Moreover, unstable matrix tethering can lead to poor cell attachment and weak engagement of cell adhesions. To address this, we developed StemBond hydrogels, a hydrogel in which matrix tethering is robust and can be varied independently of stiffness. We validate StemBond hydrogels by showing that they allow greater stability of the extracellular matrix layer, and thereby improve cell adhesion to the substrate for both mouse and human pluripotent stem cells. StemBond hydrogels further provide an optimal system for culturing mouse and human pluripotent stem cells in self-renewal conditions, including in minimal media conditions. We show how soft StemBond hydrogels modulate stem cell function, partly through stiffness-sensitive ERK signalling. Our findings underline how substrate mechanics impact mechanosensitive signalling pathways regulating self-renewal and differentiation, indicating that optimising the complete mechanical microenvironment will offer greater control over stem cell fate specification.

Decellularized Normal and Tumor Scaffolds for Cancer Organoid Cultures as a Model of Colorectal Peritoneal Metastases

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Peritoneal metastases (PM) from colorectal cancer (CRC) are associated with poor survival. The extracellular matrix (ECM) plays a fundamental role in modulating the homing of CRC metastases to the peritoneum. The ECM is a powerful regulator of cellular homeostasis, proliferation, migration, differentiation, and spatial organization patterning, thus favoring the development and the spreading of PM metastasis in the peritoneal cavity.

Studying nanomechanical properties of tissue microenvironment provides better insight into the differences of ECM derived from normal and neoplastic tissue and derived from different patients. This will allow in the future for early diagnosis of cancer development. Additionally, the possibility to isolate natural decellularized ECM and at the same time preserve both the 3D dECM architecture and the biochemical properties allows to create scaffold for cell culture, enabling the development of more physiological cancer models.

In this work we studied ECM samples by atomic force microscopy (AFM) in indentation mode. The measurements were performed with the use of custom large colloidal probe. This new approach aims at creating AFM tip with customized spring constant and sphere radius, more suitable for studied sample. We observed changes in stiffness between normal and neoplastic ECM from 5 different patients, with a trend towards increase stiffness for neoplastic samples. Moreover, the stiffness of normal ECM was found to correlate with the age of the patients. The results of nanomechanical experiments coupled with standard biological techniques provided a clearer picture of cancer metastasis.

AFM turned out to be a powerful and versatile tool for assessing the nanomechanical properties of biological samples. Our results confirm that AFM measurements on ECM can be used as in diagnosis of peritoneal metastasis, supplemented with other techniques.

Lorenc E, Varinelli L, Guaglio M, Brich S, Zanutto S, Belfiore A, Zanardi F, Iannelli F, Oldani A, Costa E, Chighizola M , Minardi SP, Fortuzzi S, Filugelli M, Garzone G, Vecchi M, Pruneri G, Shigeki K, Baratti D, Cattaneo L, Parazzoli D, Podestà A, Milione M, Deraco M, Pierotti MA and Gariboldi M, Decellularized Normal and Tumor Scaffolds for Cancer Organoid Cultures as a Model of Colorectal Peritoneal Metastases, [Preprint], <https://doi.org/10.1101/2021.07.15.452437>.

Matrix stiffness contributes to efficient oligodendrocyte differentiation altering chromatin accessibility

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Oligodendrocyte precursor cells (OPCs) are mechanosensitive cells since their biology is influenced by mechanical cues. We demonstrated that differentiation of OPCs *in vitro* is enhanced by substrates compliant with the rat brain (6.5 kPa). Recently, it was demonstrated that multiple sclerosis (MS) patients present an exacerbated brain softening compared with age and gender-matched controls. Taken together, changes in mechanical properties might impact cellular processes of OLs, with relevance in the context of MS. Little is known about the molecular mechanisms underlying the modulation of OL differentiation by mechanotransduction. Yet, some studies suggest that mechanical forces impact chromatin state during OL differentiation. Compressive forces increase trimethylation of lysine-9 residues of histone-3 (H3K9me3) that is associated with OL differentiation and maturation. Conversely, tensile forces enhance histone deacetylation and increase OL differentiation.

In this work, we will assess the molecular mechanisms involved in OL differentiation modulated by substrate stiffness through chromatin changes and transcriptional regulation.

Here, we used the assay for transposase-accessible chromatin with sequencing (ATAC-seq) in mouse primary OLs differentiated on substrates with distinct rigidity to assess chromatin accessibility. We used *de novo* motif analysis on more accessible regions to identify candidate transcription factors (TF) that may bind in differentially accessible chromatin sites. Then, we assessed Sp1 activity regulated by mechanical forces and its role on OL differentiation.

Differential analysis of ATAC-seq peaks revealed a more compacted chromatin (higher heterochromatic content) in OL differentiated on compliant substrates (6.5 kPa) when comparing with stiffer (glass) or softer conditions (2.5 kPa). These results corroborate previous reports demonstrating the enhancement of heterochromatin on OL differentiation enhanced by compressive or tensile forces. HOMER motif-discovery tool identified Sp1 as the best match among known TF motifs in all conditions. Afterwards, we assessed Sp1 mechanosensitivity through its location. We verified a higher nuclear translocation on OLs cultured on compliant substrates when comparing with those on stiffer or softer conditions after 3 days of differentiation. Indeed, Sp1 activity was higher at day 3 of differentiation. Moreover, we inhibited Sp1 during OL differentiation and observed a decrease of *Mbp* and *RhoA* expression. Additionally, we assessed the role of Sp1 and stiffness on genes involved in OL differentiation which were upregulated on glass (*Atg3* and *Dmtf1*) or on compliant substrates (*Ehmt1*) using qRT-PCR. We corroborate ATAC-seq results for these genes and Sp1 has a role in its expression. Our results emphasize the importance of culture conditions for epigenomic studies and reveal that chromatin state is a critical mediator of mechanotransduction.

The impact of biomechanical properties and glycosylation pattern on bladder cancer invasiveness

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Cancer is one of the leading causes of death in developed countries, and its most dangerous aspect is its ability to metastasize. Therefore, the key challenge of modern oncology is the development of a better model of cancer invasiveness and the determination of the characteristics of neoplastic cells that allow easy determination of their invasive potential.

We conducted our research on non-malignant HCV29 and cancer T24 and HT1376 bladder cancer cell lines. The biomechanical properties of cells were carried out using atomic force microscopy (AFM), and their cytoskeletal structure was visualized using confocal microscopy. To determine the effect of glycosylation patterns on cancer cells, we conducted experiments using lectin-coated substrates (DBA; LCA; PHA-L and WGA), with specific interactions with various sugar residues. The invasive potential we analyzed using wound healing assay, single-cell migration assay and transmigration through transwells covered with extracellular matrix (ECM).

Our research demonstrated that cancer cells are more compliant than reference, non-malignant cells. However, more invasive T24 cells showed higher stiffness than HT1376 cells, which was attributed to the organization of actin filaments. Cell morphology, assessed through fluorescent images of cell cytoskeleton in T24 cells, revealed the mesenchymal character of these cells, while HT1376 cells remained epithelial cells. Lectins significantly reduce cancer cell proliferative and migratory activity due to inhibiting sialic and GlcNAC residues.

To conclude, even though normal bladder cells are stiffer than cancer cells, there does not have to be a further decrease in elasticity with the further development of cancer. It seems that sialic and GlcNAC residues promote the invasiveness of bladder cancer. 3D cancer models will be considered in our further research because they better reflect the tumour microenvironments.

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Substrate elasticity does not affect DNA methylation changes during germ layer differentiation of induced pluripotent stem cells

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Elasticity of biomaterials has been suggested to contribute to cell-fate decisions during cellular differentiation. However, only few studies addressed the impact of stiffness on directed germ layer differentiation of induced pluripotent stem cells (iPSCs). Furthermore, it is largely unknown if effects of substrate elasticity are also reflected on epigenetic level or if they rather resemble a transient response.

In this study, we differentiated three human iPSCs lines on polydimethylsiloxane (PDMS) substrates of different stiffness (0.2 kPa, 16 kPa, and 64 kPa) or tissue culture plastic (TCP; 1-2 GPa) toward ectoderm, endoderm, and mesoderm. Germ layer differentiation of iPSCs could be induced on all substrates at similar levels according to immunofluorescence and RT-qPCR of marker genes. Only GATA6 and SOX17 revealed significantly higher up-regulation during endodermal differentiation on softer substrates. Genome-wide DNA methylation (DNAm) analysis with EPIC Illumina Bead-Chip technology showed profound and highly reproducible epigenetic modifications during differentiation of iPSC toward endodermal, mesodermal, and ectodermal differentiation. Notably, these DNAm changes were hardly affected by the substrate stiffness. Pairwise comparisons of iPSCs that were differentiated on PDMS of 0.2 kPa, 16 kPa, or 64 kPa did not reveal any significant differences. In contrast, when we compared differentiation on PDMS and TCP there were significant differences in DNA methylation patterns. Particularly during mesodermal differentiation 26159 CG dinucleotides (CpGs) became less methylated on PDMS *versus* TCP. These CpGs were significantly enriched in genes that were classified to gene ontology of cytoskeleton organization and cellular adhesion.

Taken together, elasticity of different PDMS substrates does not affect DNA methylation during differentiation of iPSCs, indicating that this parameter is not suitable to direct cell-fate decisions during early germ layer differentiation. On the other hand, biomaterials need to be critically considered since there were clear epigenetic differences upon differentiation on either PDMS or TCP.

Micromechanics of bladder cancer and fibrosis

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Modification of the extracellular microenvironment represents a hallmark of cancer, as both biochemical and biophysical features of the extracellular matrix (ECM) are modified during tumorigenesis. Among the different ECM characteristics, stiffness plays an important role on several key aspects of tumor growth and invasion (i.e., durotaxis). In this preliminary study, we aimed to establish mechanical fingerprints of the tissue layers of healthy bladder, and those associated to fibrotic bladder and bladder cancer.

For this purpose, rat bladders were isolated from untreated, x-ray irradiated and orthotopic tumor-bearing animals (bladders instilled with AY-27 cancer cell line). Tissue stiffness was measured using Atomic Force Microscopy and the Piuma nanoindenter (Optics11, Amsterdam). Micromechanical maps of the bladder wall in health and disease were associated with histological analysis, ultrasound and photoacoustic *in vivo* imaging.

We found that the healthy bladder wall was a mechanically inhomogeneous tissue. Therefore we investigated the existence of a correlation with the stiffness distribution and the different anatomical bladder layers. We observed a gradient of increasing stiffness from the internal to the outer layers: the urothelium (Young's modulus (YM) around 20 kPa); to the lamina propria (YM 100 kPa); which gradually decreased when reaching the outer layer, the muscle layer (approx. 70 kPa). Using the stiffness map of the healthy bladder as reference, we observed stiffening in fibrotic tissues, characterized by a YM up to 300 kPa that correlated with increased deposition of dense ECM. In the model of orthotopic tumor there was a gradient in the mechanical properties of the tumor-bearing bladder, with the maximum stiffness measured in bladder wall below the tumor (YM 200-300 kPa), which gradually decreased with increased distance from the tumor, reaching YM 20-50 kPa at the maximum distance from the tumor. For both pathological conditions the stiffness gradient from urothelium to muscle was maintained, and appreciated by the two different nanoindentation techniques.

This study highlights the intrinsic mechanical heterogeneity of the different bladder layers, laying out eventual hints for bladder reconstruction purposes. By providing high resolution micromechanical maps, we here reported an alteration of the mechanical properties of bladder tissue in presence of fibrosis and tumor. Such mechanical fingerprints can pave the way for future clinical diagnostic and prognostic tools.

Mechanotransduction impairment in galactosylceramidase deficient fibroblasts

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Cells sense their physical surroundings through mechanotransduction, by translating mechanical forces into biochemical signals. In turn, these signals can influence the cellular and extracellular structure. These mechanosensitive feedbacks modulate cell functions and are crucial in both physiological and pathological conditions.

Krabbe disease (KD) is a genetic rare lysosomal storage pathology causing progressive nervous system damage. The involved gene in KD codifies for the galactosylceramidase (GALC) enzyme. Such gene defect causes the accumulation of toxic lipids and leads to devastating myelin loss. To date, potential treatments are still under investigation, but their efficacy appears to be insufficient to bring back homeostasis. Probably because several molecular pathways and organelles are involved, and a clear picture of intracellular signalling involvement is not completely clear.

Therefore, our research focuses on the identification of new molecular players in the KD pathogenesis. Here, we study mechanotransduction and migration processes in primary fibroblasts collected from the twtcher (TWI) mouse, a KD murine model.

By time-lapse microscopy, we investigate single-cell and collective migration. We evaluate the development and the expression of FAs intracellular components, by immunocytochemistry and western blot. We look at the crosstalk between the mechanotransduction and the autophagy process. We study the presence and localization of Neighbor of BRCA1 gene 1 protein (NBR1), the protein needed for FAs recycling and turnover, and we test whether RAPA treatment rescue the impaired TWI fibroblasts responses.

We discover an impairment in TWI fibroblasts' mechanotransduction. These cells show single cell and collective migration delay, increased focal adhesions (FAs) number per cell, reduced adhesion junction proteins, aggregated autophagy effector NBR1. We show that rapamycin (i.e. autophagy activator) administration rescues the migration behaviour and partially normalise FAs and NBR1 levels.

Overall, the mechanosensing impairment in TWI fibroblasts correlates with autophagy. We finally suggest that mechanotransduction impairment in TWI fibro may be linked to the lipid raft' disorganization, induced by GALC-deficiency in cell membranes, and the consequent disruption of intracellular signal transduction.

High frequency microcantilever-based sensing of viscoelasticity of fluids

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We present a new sensing platform to measure rheological properties of liquids, in real-time, at high frequencies (typically in the range of 50-300 KHz), with low noise and high sensitivity. This system consists of a microcantilever excited by a piezoactuator, working as a Phase-Locked Loop (PLL) with an user-defined fixed phase $\Delta\phi$ between the excitation and deflection signals. The deflection of the cantilever is demodulated by a reference signal and the phase component (Q -signal) is used as the error-parameter fed into a PI-controller which continuously adjusts the oscillation frequency to keep the phase at the imposed value. Rheological properties of fluids, or any changes to them, can be detected by measuring the microcantilever oscillation frequency and amplitude.

This system was experimentally characterized using Newtonian fluids, and analytical and numerically models were developed and validated against the experiments. The analytical model consists of imposing the condition $Q = 0$ in the steady-state response of the system, which is equivalent to the condition $\omega_{osc}\tau_{tot} + \phi_{CT} - \Delta\phi = -\left(\frac{\pi}{2} + n\pi\right)$, with $n = 0, 1, \dots$, from where the oscillation frequency ω_{osc} and I -signal are obtained (see Figure 1). ϕ_{CT} is the phase of the cantilever, which contains the rheological properties of fluid, and τ_{tot} is the total delay mainly due to the propagation of the acoustic waves through the cantilever holder.

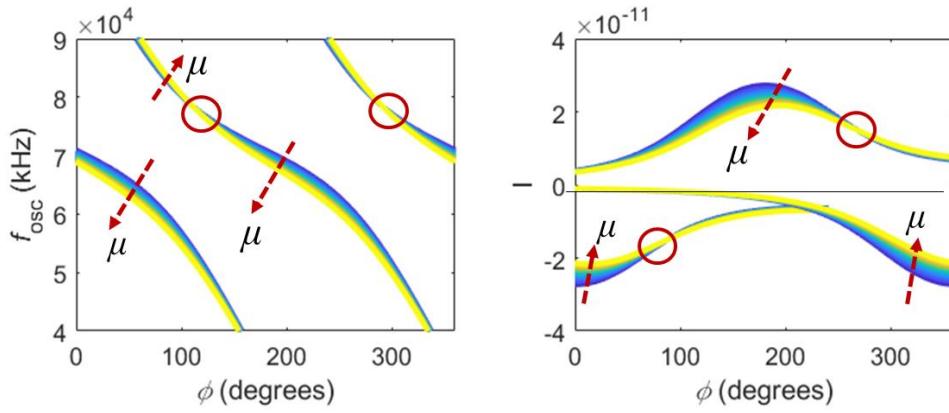


Figure 1: Modelled dynamical response of the microcantilever in a Newtonian fluid. Increasing viscosity μ of the liquid changes the frequency and amplitude responses, as indicated by the red arrows. The red circles highlight phase points whose response is insensitive to μ .

The characterisation and modelling can be extended to incorporate viscoelastic non-Newtonian fluids. In this case, the frequency-dependent elastic modulus $G'(\omega)$, viscous modulus $G''(\omega)$, and complex viscosity $\eta^*(\omega)$ can be determined from the experimentally measured frequency and amplitude responses.

This system can be used to study high-frequency viscoelastic properties of biological fluids, cells and tissues. The high frequencies of the cantilever (easily manipulated through mode of oscillation or geometry), the miniaturized dimensions, the possibility of using small amounts (μL) of liquid, and the stable frequency response are advantages not available in conventional microrheology.

Synergy in Multicomponent Biopolymer Mixtures

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There is a growing desire to design complex engineered tissue constructs for regenerative medicine purposes, as well as further knowledge of how cells respond to specific physical or chemical signals from their environment. Generally, these constructs consist of a single biopolymer component encapsulating cells with the environment acting as a mimic for certain aspects of native physiological conditions. Properties such as material stiffness, degradability, or stress-relaxation have been shown to be important to cell behaviours. In this work we design a two component mixture of cross-linkable hyaluronic acid and fibrin to promote cell viability, interactions with the matrix, and an eventual aim towards chondrogenesis.

Hyaluronic acid is a glycosaminoglycan which is an essential component of cartilage. The fixed negative charges provide an osmotic pressure which confers on the tissue its large compressive resistance. Fibrin, meanwhile, is a fibrous protein which is involved in blood clotting and wound healing. Fibrin's hierarchical structure leads to a strain stiffening behaviour whereby a network of fibrin polymers becomes stronger the more deformation is applied. We aim to mimic both the compressive resistance and the mechanical strength of cartilaginous tissue by combining these materials in an interpenetrating polymer network.

To this end, we have measured the rheological properties of mixtures of these materials and compared them with single component gels. Our results indicate a strong synergistic effect where a relatively low concentration of hyaluronic acid leads to a 5-fold increase in the stiffness of the composite material. We see that when we increase the concentration of hyaluronic acid, these synergistic effects disappear which we attribute to a disruption of fibrin formation due to the steric hindrance of the hyaluronic acid. Finally, we have performed live/dead assays on human mesenchymal stromal cells cultured in these hydrogels, showing that the viability of cells in composite materials greatly exceeds those cultured in pure fibrin networks.

Developing an *in vitro* innervated skin model with directional neurite guidance using Anisogel

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The free nerve ending of peripheral sensory neurons may degenerate in some patients suffering from neuropathic pain conditions. The complex nature of the sensory neurons innervating the skin renders them challenging for research. Tissue-engineered innervated skin models allows us to emulate the *in vivo* relationship between sensory neuron and epidermal cells, and study the underlying mechanism of their interaction. Such models are advantageous over traditional monolayer cultures e.g. as they could replicate the stratified epidermis, a feature lacking in monolayer *in vitro* culture. Tissue engineered skin models allow for a detailed study of nerve morphology and function in a controlled reproducible system.

Studies replicating the interaction between sensory neurons and keratinocytes *in vitro* often utilize neuroactive compounds, secreted in part by either cell types. On the other hand, nerve regeneration studies have directed nerve outgrowth using various topography cues, nanofiber/tube scaffold or laminin.

Our aim is to develop an *in vitro* 3D innervate skin model using Anisogel, which provides a cell adhesive environment and directional guidance for the growing neurites in the form of topography cues. The surrounding synthetic hydrogel backbone cross-links and degrades enzymatically, providing a soft, open network which is critical for sensitive nerve cells, as this setting better replicates the mechanical properties of native nerve tissue than other approaches.

Whole murine dorsal root ganglia (DRG) were encapsulated in an anisotropic PEG (polyethylene glycol) based hydrogel modified with fibronectin. Rod shaped microgels are dispersed within the bulk of the hydrogel matrix, thus forming the Anisogel. The microgels, aligned in the preferred direction using an external magnetic field, are fixed in their position upon gelation of the surrounding hydrogel. The hydrogel containing a whole DRG was cultured for a period of 7 days. The neurite growth was assessed by immunostaining with TUJ1, the microgel stained with Rhodamine, and imaged with confocal microscopy. For better visualization of neurite outgrowth, fusogenic liposomes carrying eGFP-mRNA are used to induce GFP expression in the cytoplasm of the DRG neurons. This will allow us to observe the formation of the model over time. Neurite growth was quantified using Vaa3D, an open-source visualization and analysis software, and custom written software in Python.

The presence of aligned microgels influenced the growing direction of the neurite endings, in the preferred vertical direction within 7 days of culture *in vitro*. We were able to direct neuronal outgrowth not only in a horizontal, but also in a vertical direction, which is essential for the generation of an innervated skin model. The full system could provide a physiologically relevant model, that can be expanded to include induced pluripotent stem cell-derived nociceptors from pain patients for the study of nerve-keratinocyte interaction in health and disease.

Effect of printing-associated hydrostatic pressure on cellular behavior during nozzle-based bioprinting

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Abstract

Introduction: Bioprinting is a powerful tool for the reconstruction of tissue-like structures. In bioprinting, not only the 3D structure but also the post-printing response of cells is of paramount importance. The bioprinting process impacts considerable mechanical stresses on the cells and may therefore elicit complex cellular responses.

Objective: Focusing on inkjet and microextrusion techniques, we investigated the short-term and mid-term effects of printing-induced hydrostatic pressure on epithelial and endothelial cells.

Materials and Methods: FEM simulation was used to determine the hydrostatic pressure and shear stress during the printing process. A setup was designed to stimulate cells with cyclic and constant hydrostatic pressure. Immediately after stimulation with hydrostatic pressure, cell viability, cell size, ATP level, mitochondrial membrane potential and cell metabolic activity were measured. In addition, the distribution of cytoskeletal components, cell-substrate adhesion and cell-cell contact were determined by immunocytochemistry after 24 hours to study mid-term effects on the labelled structures as a result of the hydrostatic pressure during printing.

Results: While the hydrostatic pressure did not impair cell viability, it triggered cell signaling that led to an increase in intracellular ATP in the case of cyclic pressure. The observed increase in the ATP level can either be attributed to the opening of ion-channels and/or mitochondrial respiration. The immunostaining results did not reveal any alterations in the epithelial and endothelial cytoskeleton after 24 hours.

Conclusion: Our results augment our understanding of the bioprinting effect on cells which can be used for the improvement of the design of bioprinters and proper parameter setting during printing.

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Adhesion and high-throughput mechanoprofiling of cancer cells and alginate microgels utilizing microfluidic devices

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Microfluidic devices are implemented in several aspects of cell studies from single cell to high-throughput analysis. Examples of this include the determination of changes in mechanical properties of cells related to the development of cancer and the use of mechanoprofiles to distinguish between diseased and healthy cells. Altered mechanical properties of cells may impact other cell properties such as adhesion, transmigration, and mobility. Microfluidics can be used to determine these properties in a high-throughput manner.

Cell and microgel mechanical properties can be deduced from their deformation when acted upon by a passive external force such as fluid shear stress or forcing the object through a constriction smaller than its diameter. The global elasticity can thus be linked to the resulting shape change. However, this can prove complicated when dealing with an unknown sample, therefore a calibration object is often used, where the elasticity of the sample is already known. In the present study alginate microgels, with varying Young's moduli fabricated using a microfluidic flow-focusing droplet generation device, and bladder cancer cells from three different stages of cancer progression (HCV29, T24, and HT1376) were compared.

Four different types of microfluidic devices were fabricated using photo- and soft-lithography. First, flow-focusing droplet generation devices were fabricated and employed to generate alginate microgels at rates 1-3kHz. The microgels were generated using four different sized alginates with four different concentrations (0.4-0.9%) creating 16 different elasticities. Two different constriction-based channels were also fabricated. These were employed to impose fluid shear stress and geometric compression thus mimicking cells in the blood stream and as they traverse the blood vessel layer, respectively. The result of the microfluidic mechanical analysis was compared to AFM measurements using the same alginate microgels and cells. The adhesive properties of cells under microflow were also investigated and compared to static and microflow conditions. In this instance, the surface of the microfluidic channel was functionalized with four different lectins (WGA, DBA, LCA, and PHA-L) and the selective adhesion of the cells was explored.

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Immunomodulatory proteins from pathogenic bacteria alter the mechanophenotype of human blood platelets

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Background:

Systemic infection with opportunistic human pathogen the Gram-positive *Staphylococcus aureus* can lead to infective endocarditis (IE) and disseminated intravascular coagulopathy (DIC). In blood, *S. aureus*, their secreted or surface-localized immunomodulators proteins directly and indirectly (e.g., via serum proteins) with platelets. Within this context, the cell biology of host-pathogen interaction has attracted much attention. However, whether these interactions alter the biophysical properties such as viscoelasticity and its significance in platelet margination during blood circulation remains elusive.

Aims:

To assess the changes in viscoelastic properties (i.e., biomechanical deformation) and associated activation states of single human platelets induced by immunomodulators proteins of *S.aureus* proteins using high-throughput functional mechanophenotyping.

Methods:

Functional mechanophenotyping of single platelets was performed by high-throughput, real-time 1D-imaging fluorescence, and deformability cytometry (RT-FDC). Human platelets were incubated with *S. aureus* proteins: phospholipase C (Plc), major autolysin (AtLA-1), extracellular adherence protein (EapD₃D₄ domain), chemotaxis inhibitory protein (CHIPS), and formyl peptide receptor-like inhibitory protein (FLIPr). Changes in platelet deformation, their size, and activation were detected in RT-FDC, simultaneously using fluorescent molecular markers. Fluorescence flow cytometry and microscopy were to assess the changes in platelet cytoskeletal content and its subcellular distribution. Impact on platelet margination, thrombus formation, and microarchitecture was evaluated *ex vivo* under arterial shear in a microfluidic setup.

Results:

EapD₃D₄ and CHIPS lowered platelet deformation and induced platelet activation (CD62P⁺) simultaneously, whereas AtLA-1 induced lower deformation in the absence of platelet activation, with a concomitant increase in F-actin content. While both Plc and FLIPr failed to activate platelets, we observed Plc-induced viscoelastic softening without changing F-actin content.

Conclusions:

Single platelet mechanophenotyping revealed *S. aureus* proteins selectively alter platelet deformation and activation. Furthermore, this significantly influences platelet margination at the vessel wall, the kinetics of thrombus formation, its size, and stability. Intriguingly, some individuals also exhibit a heterogeneous mechano-functional response to identical proteins. The *in vivo* relevance of pathogen protein-induced effects on viscoelastic biomechanical properties of platelets and other peripheral blood cells during systemic infection by pathogenic bacteria warrants further investigation.

Vimentin intermediate filaments mediate cellular responses to substrate stiffness and substrate viscoelasticity

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The ability of cells to take and change shape is a fundamental feature underlying development, wound repair, and tissue maintenance. Central to this process is physical and signaling interactions between the three cytoskeletal polymeric networks: F-actin, microtubules, and intermediate filaments (IFs). Vimentin is an IF protein that is essential to the mechanical resilience of cells and regulates cross-talk amongst the cytoskeleton, but its role in how cells sense and respond to the surrounding extracellular matrix is largely unclear. To investigate vimentin's role in substrate sensing, we designed polyacrylamide hydrogels that mimic the elastic and viscoelastic nature of *in vivo* tissues. Using wild-type and vimentin-null mouse embryonic fibroblasts, we show that vimentin can enhance or hinder cellular traction forces depending on the stiffness of the substrate. While cells lacking vimentin can still spread on elastic substrates, vimentin is critical for fibroblast spreading on viscoelastic substrates. Our results provide compelling evidence that the vimentin cytoskeletal network is a physical modulator of how cells generate and transfer forces, which is central to how cells sense and respond to their local environment.

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Plastic adaptation of the retinal pigment epithelium alters material properties of the monolayer

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Simple epithelia delineate cavities in the body ensuring firm barrier and selective transport of molecules. Their function is ensured by properties emerging from the ability of each cell to interact with their neighbours. These intercellular interactions permit the formation of a continuous sheet of mechanically linked entities, making epithelia capable of withstanding and propagating forces. Here, cell morphology and organization are indicative of tensions and pressures of the sheet with preferred stable configurations like an ideal honeycomb-like arrangement.

In the retina, the simple cuboidal pigment epithelium (RPE) is essential for the proper function of the light-detecting photoreceptor cells. In the area of highest photoreceptor density (macula in human), the RPE monolayer shows a homogeneous tiling, consisting of pentagonally and hexagonally shaped cells. In aging and disease, this homogenous tiling becomes highly disorganized featuring cytoskeletal defects and increased heterogeneity of cell shapes and sizes. Due to its postmitotic nature, the RPE compensates natural cell loss by hypertrophy and cellular reconfiguration to preserve monolayer integrity. This adaptive strategy may affect the monolayer mechanics and thus fundamental function of the tissue.

Using a murine model, we characterized and quantified age-related changes of monolayer organization and for the first time defined the nature of local tiling defects. These comprise of hypertrophic multinucleated cells with an area which exceeds the average cell size by at least three standard deviations and with at least eight neighbouring cells of various sizes. To quantitatively analyse the significance of such defects for RPE mechanics, we are establishing an *in-vitro* model of iPSCs-derived RPE cells on hydrogels. To induce RPE packing defects, we challenge mature low-proliferative iPSCs-derived RPE monolayers with large-scale apoptosis mimicking age-related cell death. With nanoindentation, traction force and monolayer stress microscopy, we evaluate the mechanical shift resulting from the accumulation of defects in the monolayers. Furthermore, cytoskeleton imaging and photoreceptor outer segment internalization assay will connect the acquired mechanical equilibrium with the epithelial phenotype and function.

In analogy to plastic deformation of material, age-related accumulation of mechanically challenging cellular configurations, may increase the RPE fragility to stress and contribute to the development of degenerative diseases.

The lipid kinase, PIP4K2B, controls cell mechanical outputs impacting on heterochromatin dynamics through UHRF1

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PIP4K2B is a phosphoinositide kinase mainly located in the nucleus where it phosphorylates PI5P to PI(4,5)P₂ and controls transcriptional outputs by regulating epigenetic signalling complexes. PIP4K2B is highly expressed in cancer and its expression often correlates with worse patients' outcome. Moreover, evidences indicate PIP4K2B as necessary for the growth of tumours in *TP53*^{-/-} mice, and for the function of the immune response suppressors regulatory T cells. For all these reasons, small molecular weight compounds targeting this enzyme have been developed recently to treat cancer patients. Despite the deep knowledge about PIP4K2B involvement in cellular outcomes, nothing is known about its role in cell migration or mechanotransduction.

Our data indicate that PIP4K2B protein levels are altered in cell seeded on soft substrates. In addition, direct depletion or inhibition of this phosphotransferase strongly controls mechanical features of epithelial cells impacting on heterochromatin. In fact, RNA seq analyses of cells seeded on soft substrates or depleted of PIP4K2B results in a highly conserved signature of genes involved in chromatin remodelling. In particular, depletion of PIP4K2B downregulates the levels of UHRF1, a protein that allows binding of the histone methyltransferase SUV39H1 to H3K9me3 sites, controlling heterochromatin status during cell cycle. This leads to impaired levels of H3K9me3, which drive alterations in chromatin compaction, nuclear envelope tensional state and changes in nuclear polarity mimicking a soft-like status of the cells. This altered mechanics of the nucleus is mirrored by YAP nuclear extrusion, changes in cytoplasmic and cell membrane mechanical dynamics, followed by defects in cell spreading and cell motility. Altogether, our data suggest that PIP4K2B is a novel player in the mechanoregulation process and that potential therapeutic approaches targeting its activity could impact on cell mechanical responses and capacity to migrate.

Combining Metal Induced Energy Transfer and Atomic Force Microscopy to Probe the Mechanoresponse of a Focal Adhesion

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Focal adhesions function as anchoring points to the extracellular matrix, yet also enable cells to sense and exert forces on their environment [1]. Focal adhesions are complex structures consisting of a multitude of different proteins.

Despite the important role of the focal adhesion complex in cellular adhesion, its structure and mechanoresponse remain difficult to resolve [2]. Knowing the exact position of the proteins in the focal adhesion complex under strain is necessary to understand their working principle.

For a detailed analysis of the focal adhesion architecture coupled with force response, we need a method to measure small distances while manipulating the cells with an AFM. To meet this challenge, we couple AFM techniques [3] with Metal Induced Energy Transfer (MIET) [4] to resolve positions at the nanoscale level. Here we show an initial analysis of the interplay between focal adhesion associated actin and force.

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Mechanical phenotyping of fibroblasts to diagnose visceral myopathy

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Gastrointestinal motility disorders are a major medical challenge ranging from common and generally benign conditions, to rare and potentially life-threatening diseases [1]. Chronic intestinal pseudo-obstruction (CIPO) represents the most severe form of motility disorder and is characterized by failures in the propulsion of the intestinal content due to inefficient peristalsis [1]. Visceral myopathy (VSCM) represents the myogenic form of CIPO, a rare genetic-based gastrointestinal motility disorder clinically characterized by intestine, bowel, bladder, and uterine dysfunction [2]. The most common genetic cause of VSCM is a mutation in the smooth muscle actin gene (ACTG2) [3], leading to a reduction of muscle function [4].

The pathology has a deep impact on life quality: currently no pharmacological treatment is available [5], and individuals endure prolonged hospitalizations and surgical interventions, surviving with compromised quality of life. Therefore, advancements in the diagnostic strategy represent a pressing unmet medical need. A rapid diagnosis is crucial for a correct management of the patient treatment, and a breakeven point for patients and families.

Considering the crucial involvement of the visceral smooth muscle actin in VSCM and the macroscopic symptoms related to the loss of peristalsis, we present a mechanical study carried out on a set of primary dermal fibroblasts from VSCM-affected patients, and a set of control primary dermal fibroblasts, to highlight *in-vitro* live cell aspects potentially able to join clinical and genetic features in supporting and orienting VSCM diagnostic and therapeutic pathways. Actuating forces of the cells transmitted to the substrate were captured by a recently developed reference-free confocal traction-force microscopy platform [6]. We found a significant reduction in forces for the cells carrying the ACTG2 mutation compare to the control fibroblasts.

This work indicates that dermal fibroblasts represent a reliable and manageable model for the study of VSCM, that could be potentially useful to set-up specific diagnostic tests based on cell biomechanics and biophysical approaches. Furthermore, we demonstrate that cell traction force could be a suitable parameter to discriminate between fibroblasts of VSCM patients and controls, with potential benefits on the diagnostic and the drug discovery strategies.

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Light-activation of Src in epithelial cells reveals its mecanosensitive behavior

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Over the past few years, several studies have uncovered a role for mechanical signalling in tumorogenesis. During cancer progression, cells will acquire new biomechanical functions, which affect the way they interact with their environment. Such biomechanical changes possibly provide them an advantage on their normal counterparts to invade the surrounding tissues. The interactions between transformed cells and their mechanical environment are not fully understood and need to be explored. In this context, we aim at understanding the alterations of cellular mechanosensitivity on the onset of tumorogenesis.

We focus on the oncprotein Src, found to be overexpressed in many cancers and involved in their metastatic spread. Our approach consists in characterizing the biomechanical response of single Src-transformed cells for different substrate rigidities. We choose optogenetics to precisely control in time and in a reversible way the Src activation in cultured epithelial cells. In parallel, we characterize the mechanical state of the transformed cells using Traction Force Microscopy for cells deposited on fibronectin-coated polyacrylamide (PAA) gels of given rigidity.

We first showed that isolated cells that are Src-activated spread while pulling more on their substrate. Interestingly, this increase in cell contractility does not rely on the activation of the Rho pathway. This change in the mechanical phenotype is accompanied by a huge restructuration of the cytoskeleton: reorientation and increase in the number of cell focal adhesions, and reorganization at the cell periphery of the stress fibers network. Using PAA gels of different Young's moduli, we found that the gain in spreading and contractility during Src activation depends on substrate rigidity. Of note, we identified a particular rigidity around 10 kPa for which both the gain in area and in force associated to Src-activation are maximal. Our work depicts the mechanical phenotype acquired by Src-transformed cells and reveals how cell mechanosensitivity is impacted.

The Weakness of Senescent Dermal Fibroblasts

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Skin is the largest human organ with easily noticeable biophysical manifestations of aging. As human tissues age, there is chronological accumulation of biophysical changes due to internal and environmental factors. Skin aging leads to the loss of dermal matrix integrity via degradation and decreased elasticity. The mechanical properties of the dermal matrix are maintained by fibroblasts, which change their properties during replicative aging. While the secretory phenotype of senescent fibroblasts is well studied, little is known about changes in the fibroblasts biophysical phenotype. Here, we compare biophysical properties of young versus proliferatively aged primary fibroblasts via fluorescence and traction force microscopy, single-cell atomic force spectroscopy, and micro- rheology of the cytoskeleton.

Results show senescent fibroblasts have decreased cytoskeletal tension and myosin II regulatory light chain phosphorylation, in addition to significant loss of traction force. The alteration of cellular forces is harmful to the process of building and maintaining extracellular matrix, while decreased cytoskeletal tension can amplify epigenetic changes involved in senescence. Further exploration of these mechanical phenomena provide possibilities for unexplored pharmaceutical targets against aging.

Development of an atomic force microscopy system for long-range, high-speed spectroscopy on cells

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The formation of membrane tethers during the leukocyte adhesion cascade is a fast process in which tens-of-micrometers-long tethers are formed at high velocities due to the high shear rate of the bloodstream [1], [2]. Thus, a quantitative understanding of the tether formation on leukocytes requires force measurements in a long-range (up to 100 μm) and biologically relevant velocities (in the order of millimeters per second).

Although tethers' formation has been previously studied by atomic force microscopy (AFM), typical setups do not satisfy the needed length and velocity criteria mentioned above. The maximum length of a force vs. indentation curve is limited to the movement range of the piezo in the vertical direction. The typical vertical range is $\sim 15 \mu\text{m}$ in most cellular AFM systems, up to $\sim 200 \mu\text{m}$ in some cases, but the velocity at which force curves are acquired is generally lower than 100 $\mu\text{m/s}$ [3].

This work presents a prototype of an AFM system capable of acquiring force curves at 2 mm/s with a range of 60 μm . It is based on the commercially available PS-NEX system from RIBM. We implemented a new sample stage to acquire force curves by moving the sample using a fast levered piezoelectric actuator. The AFM head is mounted on the stage of an inverted spinning-disk confocal microscope to track cells during the force spectroscopy process. The system is compatible with ultrashort cantilevers, like those developed for high-speed AFM, to minimize the high viscous drag forces induced by the liquid at high velocities. Therefore, the system will allow high-speed force spectroscopy directly on living cells.

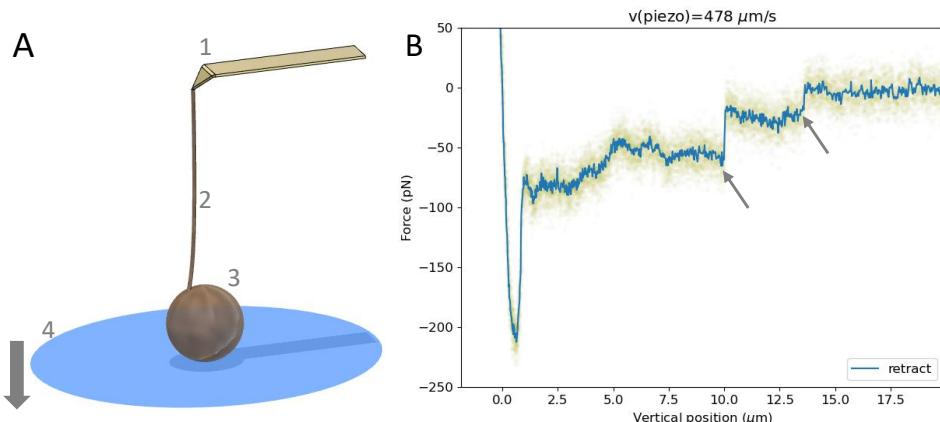


Figure 1. (A) Cantilever (1) pulling a tether (2) from a leukocyte (3) during the stage (4) retraction. (B) A 20 μm long force curve at 478 $\mu\text{m/s}$ exhibiting two tethers (marked with arrows).

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Mechanobiology of endothelial senescent cells

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Ageing is the major risk factor contributing to many disorders, including cardiovascular diseases (CVDs) that represent the leading cause of death in the elderly population of higher-income countries (Paneni et al.; Nouman). One of the hallmarks of the ageing process is the progressive accumulation of senescent cells (Ting et al.), and the causative role of senescence in diseases and ageing has already been demonstrated *in vivo* (Baker et al.; Hashimoto et al.). During the ageing process, endothelial senescent cells (ESCs) accumulate in the vascular endothelium and alter vessels' morphology and functions enhancing thrombosis, inflammation, and increasing the possibility of developing CVDs (Jia et al.).

ESCs are characterized by enlarged morphology, metabolic changes, alterations in chromatin organization, and the activation of a pro-inflammatory secretome, named senescence-associated secretory phenotype (SASP) (Ting et al.). These cells activate different inflammatory pathways and become pro-thrombotic, and pro-atherosclerotic.

Endothelial cells are exposed to different physical stimuli: the blood flow generated wall shear stress, and the mechanical forces exerted by the underlying layers. So far, biophysical research on ESCs has mainly focused on the effects of the blood flow and the topography of the substrates on the cells (Chala et al.). However, it is well known that also the mechanical properties of the surrounding tissue strongly influence cells behaviour (Chaudhuri et al.).

This work aims at exploring the mechanical and physical signals involved in the senescence phenomenon. The objective of this research is to study the reaction of the senescent cells to substrates of different mechanical properties, e.g., hydrogels. The cells will be cultured on hydrogels with controlled and tunable viscoelastic properties, replicating the mechanical features of the vessels' basement membrane. These samples will be subjected to the flow of a bioreactor that simulate the blood shear stresses. The reaction of the cells will be studied in terms of both mechanical properties, and phenotypical characterization, analyzing the main traits of the SASP.

Understanding the mechanical properties of the endothelial senescent cells, and their mechanosensing reaction and alteration could be impactful in different applications. For instance, the mechanical characterization of the cells and their response to mechanical stimuli could help, respectively, the identification and the depletion of the ESCs during cardiovascular surgeries and stents application.

Insights into Mechanopharmacology of Aspirin on Human Blood Platelet Deformation and Clot Mechanics

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The antiplatelet drug aspirin or acetylsalicylic acid (ASA) acts on cyclooxygenase-1 by irreversible acetylation of one serine residues. This blocks the catalytic conversion of arachidonic acid to prostaglandin H₂, thus inhibiting platelet activation via thromboxane receptor. Beyond this, it is believed that non-selective acetylation by ASA also impacts the function of serum and cellular proteins, including the platelet cytoskeleton.¹ In addition, it is suspected that ASA may affect the stability of fibrin clots by increasing the clot porosity, thus accelerating fibrinolysis.^{2,3} However, thus far, the quantitative insights into the mechanopharmacological aspects governing the impact of ASA are poorly understood.

Here we report on the mechanopharmacology of ASA, on the functional mechanophenotype of single human platelets by high-throughput real-time fluorescence and deformability cytometry (RT-FDC), the stiffness of thrombi by colloidal probe spectroscopy and the bulk mechano-kinetic properties of clot formation and its lysis by rotational thromboelastometry. ASA concentrations of 5.5 μM (100 mg oral dose) and 55 μM (500 mg oral dose), which reflect drug plasma levels after administration of therapeutic doses of ASA, were used.

Non-stimulated control platelets in PRP showed deformation of 0.097 ± 0.015 (mean \pm SD, n=6 donors), which was significantly higher in comparison to the less deformable platelets at 0.068 ± 0.015 in 5.5 μM ASA and 0.059 ± 0.013 in 55 μM ASA. Under shear on collagen, platelets from ASA-treated blood formed thrombi, which were softer (5.5 μM aspirin: Young's modulus E: $2.54 \text{ kPa} \pm 0.91$ and 55 μM aspirin Young's modulus E: $2.43 \text{ kPa} \pm 0.72$) than control thrombi (Young's modulus E: $3.0 \text{ kPa} \pm 0.91$). Rotational thromboelastometry revealed, at 55 μM of ASA increased clotting time (CT: 93 s) and clot formation time (CFT: 146 s) compared to controls (CT: 54 s and CFT: 107 s). In addition, in ASA, thrombi were smaller (MCF: 57 mm) with a lower elastic shear modulus (G: 6707) and reduced thrombodynamic potential index (TPI: 28) compared to controls (MCF: 60 mm, G: 7512; TPI: 42). Furthermore, with ASA, in the presence of tissue plasminogen activator (tPA), a significantly reduced lysis onset time (LOT: 2052 s) and clot lysis time (LT: 2821 s) was observed compared to the controls (LOT: 2197 s and LT: 3011 s).

Our data show that ASA influences platelet deformation, suggesting ASA may significantly impact the platelet cytoskeleton, affecting their hemodynamic and margination properties. Furthermore, the thrombi formed under shear are softer with decreased clot density, making them susceptible to fibrinolysis. In summary, our data suggest that the pharmacological action of ASA cannot be accounted for by cyclooxygenase blockade alone, thus demonstrating the importance of these novel mechanopharmacological insights into ASA for its antithrombotic efficacy. Furthermore, a mechanopharmacological approach may offer targeted, personalized mechanomedicine for selective cardiovascular diseases from a translational perspective.

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Identifying Label-free Biomarkers for Visceral Myopathy

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Visceral myopathy (VSCM) is a rare gastrointestinal motility disorder which is characterised by impaired intestinal function and motility in the absence of mechanical obstruction. It is a severe disease which mainly affects infants and children. The main symptoms include abnormal intestinal activity, malnutrition and abdominal pain.

VSCM is a smooth muscle disorder where ACTG2 gene has been identified as the genetic cause in 40% to 50% of cases. However, there is currently no clear genetic driver which has made early diagnosis for VSCM difficult. This has led to unwanted surgical intervention used to treat patients once the disease has progressed. Thus, the need for a label-free biomarker arises for VSCM to improve diagnosis and ultimately treatment.

VSCM is characterised by impaired peristalsis, often leading to misdiagnosis of this disorder as a form of obstruction. Preliminary results show that the ability of single cells to contract and generate forces on the extracellular matrix is also impaired in VSCM patients, and we are working to convert this evidence into an assay to support early diagnosis of the disease.

We are currently implementing collagen gel contractility assays and standardising the readout to obtain a robust marker of the medical condition. The activity is being performed on primary skin fibroblasts from VSCM patients and from control patients who are affected by other diseases, not related with intestinal pseudo-obstruction.

Chitosan blended micro-grooved membranes for nerve regenerative applications

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Regenerative medicine is continuously searching for new natural and biocompatible materials that are also mechanically compliant, and possibly biodegradable. Chitosan is emerging as a promising FDA-approved biopolymer for tissue engineering thanks to its properties of biocompatibility and biodegradability. However, its exploitation in regenerative devices can be further improved by tuning its nano/microstructure and by blending it with other materials. At the same time, the critical importance of the nano/micro-structured features of the scaffold surface is overall accepted, in view of the *in vitro* and *in vivo* regenerative performance. We already demonstrated that nano/micro-gratings, ranging from ultra-small to micro topographies, are capable to direct neuronal and glial cell differentiation, polarization, and migration. Each cell type responds to specific length scales according to the specific cell features.

Our study aims to develop microstructured scaffolds for peripheral nerve regeneration, made of biodegradable and soft materials, able to be compliant with nerve mechanics. Here, we develop, by solvent casting, thin films made of chitosan and glycerol, and micro-patterned with directional geometries having different levels of axial symmetry. These membranes are optimized for their mechanical properties, and characterized by scanning probe and light microscopies, and by mechanical and degradation assays. The chitosan-glycerol membranes are tested as *in vitro* scaffolds with neuronal and Schwann cells, to study cell migration and healing, and as *in vivo* scaffolds, on the Cavernous nerve injury model in rats. Overall, we show that our chitosan-glycerol membranes show optimal physico-mechanical properties, enhance the Schwann cell healing response *in vitro* and also *in vivo*.

Our results may be exploited for the production of artificial interfaces for enhancing nerve regeneration, by promoting Schwann cell migration and nerve guidance.

Development of a three-dimensional skin model to investigate the mechanical properties of keratin intermediate filaments

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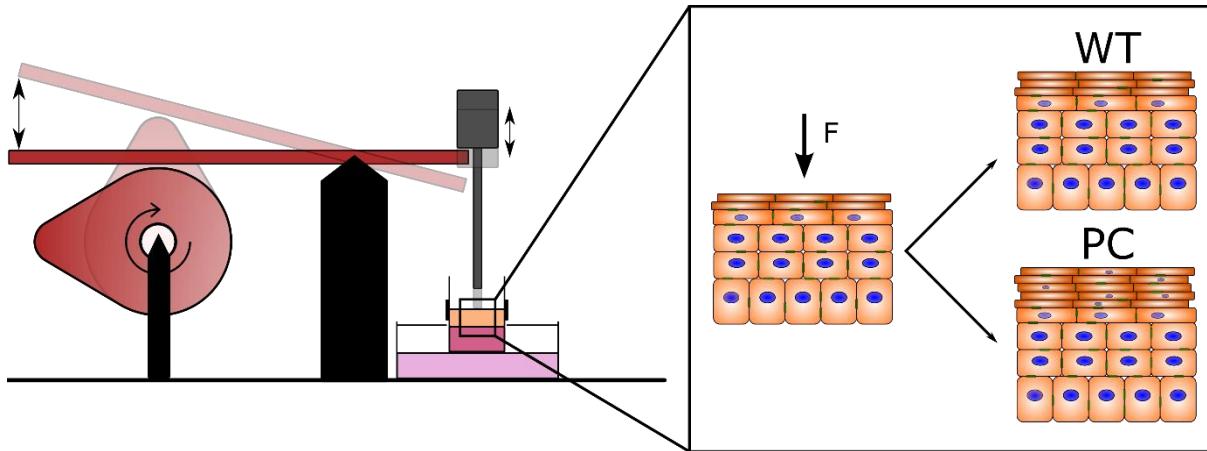


Figure 1: Graphical version of the abstract.

The epidermis is the body's first line of defense against external insults. Keratin intermediate filament networks play an important role in the organization and stability of epidermal keratinocytes by resisting forces acting on the epithelium. Mutations in keratin-coding genes lead to an impairment of the mechanical properties of the epidermis and consequently result in rare keratinopathies, which, depending on the affected keratins, show different phenotypes.

Research efforts to elucidate the mechanical properties of different keratins and the consequences of their mutations have mainly focused on experimental set-ups involving single cells or differentiating monolayers. In our work, we are implementing skin models with an epidermal compartment grown from immortalized cell lines of healthy donors as well as from patient cell lines.

We study exemplarily keratin 5 and 14-related *epidermolysis bullosa simplex* (EBS) and keratin 6, 16 and 17-related *pachyonychia congenita* (PC) as model diseases, because they show clearly distinguishable phenotypes. EBS is characterised by intraepidermal blisters whereas epidermal hyperproliferation is a hallmark feature of PC. Since these skin pathologies typically manifest in response to mechanical insults, we developed a device to apply cyclic compressive load to the skin models in a controlled manner allowing the examination of consequences of defined mechanical stress (Figure 1).

We found that both types of mutant keratinocytes are capable to differentiate into multilayered epidermal models expressing differentiation markers that are typically found in native epidermis. Furthermore, we obtained strong evidence that the applied mechanical stimulation evokes the hyperkeratotic phenotype in PC-derived epidermal models presenting a cornified layer (*stratum corneum*) that is two to three times thicker than in non-stimulated and wild-type control samples. Experiments are currently under way to examine the response of EBS-derived epidermal equivalents.

We conclude that the patient-derived keratinocytes together with the compression device provide a controllable experimental setup to investigate the perturbed mechanical homeostasis of the epidermis in keratinopathies and to get a better understanding of the contribution of keratins to the three-dimensional stability and differentiation of the epidermis.

Investigation of interaction between bladder cancer cells and lectins by AFM-based single-cell force spectroscopy

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Bladder cancer is one of the most often diagnosed cancers globally. Mortality is inextricably linked to the stage cancer reached at the moment of diagnosis. Studies on cancer progression have shown enormous mutations and molecular alterations on the cell surface, including abnormal glycosylation patterns. Several research groups have proposed the use of glycans as cancer biomarkers.

To recognize the different glycans, many techniques in molecular biology use plant lectins. Lectins are characterized by a special ability to bind various glycoproteins, and the bindings are very specific, such as ligand-receptor interactions.

This study aimed to assess the adhesion of bladder cancer cells from different stages of cancer progression, i.e. HCV29, T24, and HT1376, to lectin-coated surfaces. The adhesive properties of the cells were investigated using fluorescence microscopy and single-cell force spectroscopy (SCFS) to evaluate cell adhesion qualitatively and quantitatively. In addition, fluorescence microscopy was used to evaluate the distribution of glycopatterns on the surface of various bladder cells.

The obtained results show that cells from different stages of tumor development show a different affinity for different lectins. These findings suggest that lectins can be used to isolate the subpopulation of a specific type of bladder cancer cells based on the adhesive properties of these cells.

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Mapping tumor spheroid mechanics in confining 3D microenvironments

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Altered biophysical properties of cancer cells and of their microenvironment contribute to cancer progression. While the relationship between microenvironmental stiffness and cancer cell mechanical properties and responses has been previously studied using two-dimensional (2D) systems, much less is known about it in a physiologically more relevant 3D context and in particular for multicellular systems. To investigate the influence of microenvironment stiffness on tumor spheroid mechanics, we first generated MCF-7 tumor spheroids within matrix metalloproteinase (MMP)- degradable 3D polyethylene glycol (PEG)-heparin hydrogels, where spheroids showed reduced growth in stiffer hydrogels. We then quantitatively mapped the mechanical properties of tumor spheroids *in situ* using Brillouin microscopy. Maps acquired for tumor spheroids grown within stiff hydrogels showed elevated Brillouin frequency shifts (hence increased longitudinal elastic moduli) with increasing hydrogel stiffness. Maps furthermore revealed spatial variations of the mechanical properties across the spheroids' cross-sections. When hydrogel degradability was blocked, comparable Brillouin frequency shifts of the MCF-7 spheroids were found in both compliant and stiff hydrogels, along with similar levels of growth-induced compressive stress. Under low compressive stress, single cells or free multicellular aggregates showed consistently lower Brillouin frequency shifts compared to spheroids growing within hydrogels. Thus, the spheroids' mechanical properties were modulated by matrix stiffness and degradability as well as multicellularity, and also to the associated level of compressive stress felt by tumor spheroids. Spheroids generated from a panel of invasive breast, prostate and pancreatic cancer cell lines within degradable stiff hydrogels, showed higher Brillouin frequency shifts and less cell invasion compared to those in compliant hydrogels. Taken together, our findings contribute to a better understanding of the interplay between cancer cells and microenvironment mechanics and degradability, which is relevant to better understand cancer progression.

Brillouin microscopy for extracellular matrix viscoelasticity characterization in bladder tissues

Claudia Testi

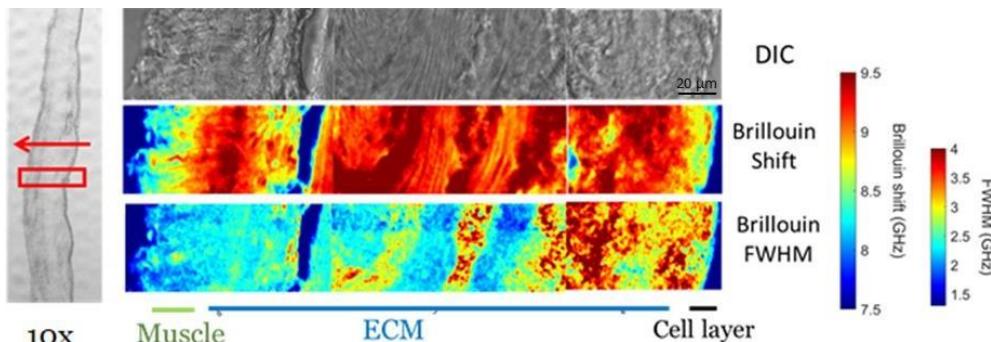
Pathological changes in tissue stiffness can be traced to altered amounts and function of its two fundamental constituents: cells and extracellular matrix (ECM). ECM in particular is a highly dynamic matrix that influences tissues behavior not only by its chemical composition but also through its mechanical properties (stiffness and viscosity). During cancer and fibrotic diseases, dysregulated matrix synthesis and remodeling takes place: thus, ECM plays a fundamental role in regulating physiological functions, but studying its mechanical properties is to date a challenging task in biophysics.

Here, we are interested in characterizing changes in ECM viscoelasticity in bladder tissues samples. Bladder is indeed an excellent example of an organ in which mechanical properties regulation is essential for its function: it has to adapt and stretch to the urine volume and undergo high elastic extension. Aging, neoplasia and metabolic diseases can lead to fibrosis and to an increase of bladder wall elasticity. Thus, tissue stiffness in bladder is a clinically relevant parameter for diagnosis and prognosis purposes.

In collaboration with the Urological Research Institute of Ospedale San Raffaele in Milan, our group in Istituto Italiano di Tecnologia in Rome measured the viscoelastic properties of murine bladder tissues by using our custom-built Brillouin Microscope. This technique allows for non-invasive and label-free imaging of mechanical properties in living tissues with sub-micron resolution.

Preliminary Brillouin maps show areas of high heterogeneity, rich in biophysical information. These data indeed demonstrate that ECM in normal and fibrotic samples of bladder have different properties both in stiffness (related to Brillouin shift) and in viscosity (related to Brillouin peak full width at half maximum).

Such mechanical information is very important and relevant for several purposes, as for label-free characterization of tissues and for the development of innovative diagnostic tools.



A wild-type murine bladder segment obtained with our Brillouin microscope. We superimpose transmitted DIC information (obtained with a 10x on the left and with a 60x objective on the right) and the Brillouin maps of stiffness (Brillouin shift) and viscosity (Brillouin peaks FWHM). Through Brillouin imaging, we can distinguish the different portions of the tissues, namely cells, ECM and muscles, with an higher contrast than the DIC and without the use of markers.

Human HSC migrate in response to human thymic stromal cells in a microfluidic model of extravasation

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The human thymus is the site of T cell maturation and development of central tolerance. Hematopoietic stem cell (HSC) derived T cell progenitors are continually recruited to the thymus from the fetal liver and bone marrow during prenatal and postnatal development, respectively. The mechanism by which HSC are recruited to the thymus is poorly understood in humans, though chemokines expressed by thymic stromal cells (TSC), including epithelial cells (TEC) and mesenchymal-like interstitial cells (TIC) have been shown to be important for recruitment in mouse models. As epithelial/thymocyte interactions are crucial for both T cell and thymus development, understanding the signals leading to HSC recruitment is essential.

We have developed a microfluidic based assay to mimic human HSC extravasation across the endothelium into the extracellular space in the presence or absence of human TSC. Briefly, microdevices were seeded with human endothelial cells to mimic an endothelium; CD34⁺ HSC were added to the endothelial channel and were allowed to extravasate over a 24-hour period before imaging. HSC demonstrated an intrinsic propensity towards migration, with detectable extravasation after 24 hours. Additionally, the trans-endothelial migration of the HSC was seen to occur via a paracellular route.

Addition of exogenous cytokines or culturing of TSC in a parallel channel significantly increased HSC extravasation. Culturing TEC or TIC in the parallel channel alone led to extravasation rates similar to, or less than, control devices, suggesting that the total thymic microenvironment is crucial for thymocyte recruitment. Together, our results suggest that chemoattractants secreted by the thymic stroma are crucial for thymus seeding and that manipulation of these signals may have the potential to increase thymocyte recruitment in a therapeutic setting.

Novel Bioreactors for Mechanical Stimulation in Respiratory Tissue Engineering

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Airway cancer is often diagnosed at an advanced stage when treatment options are limited. Autologous replacement tissue produced by the means of tissue engineering could in the future provide a new option for patients suffering from tumors in the respiratory tract. As airway tissues experience diverse mechanical stresses *in vivo* such as shear, tension or collision, they are characterized by specific biomechanical properties that also have to be matched by tissue engineered constructs. In this study, we therefore developed two different bioreactors for the *in vitro* culture of either a tissue-engineered respiratory epithelium or an engineered vocal fold replacement.

The mucus production and ciliation of the respiratory epithelium provide an important cleaning mechanism that needs a unidirectional orientation of the ciliary beating throughout the whole airway tissue to function properly. Little is known about the cues needed for ciliary orientation. To overcome this problem, we established a bioreactor system for upper airway epithelial cells, which allows us to investigate the influence of mechanical stimuli by means of shear stresses on the orientation of cilia. The established two-chamber bioreactor system consists of a bottom channel for dynamic medium supply and an upper channel for cell culture and stimulation. Both channels are separated by a PET-membrane to support the growth and differentiation of human respiratory epithelial cells (HREs). A mechanical stimulus similar to the wall shear stress in native tracheal tissue can be applied to the cells via airflow. Epithelial cultures of the bioreactor are compared to static controls using standard culture systems. Among molecular analysis of epithelial, ciliary and cell polarity marker genes via qPCR, immunofluorescence stainings are used to investigate the ciliary orientation and cell polarization. Furthermore, a live staining of the cilia with wheat germ agglutinin is carried out.

With regard to the vocal fold, we developed a bioreactor that combines stretching and vibrational stimulation. In order to match the tissue's *in vivo* characteristics, we aimed for a bioreactor allowing 20 % tensile strain and a frequency range between 100 and 300 Hz at least, with alternating stimulation patterns including resting periods. Compatible scaffolds for cell seeding include hydrogels and elastic membranes. In addition, we targeted a reusable, straightforward design that is easy to implement and to sterilize. Electrical components are designed to avoid contact with the humidified incubator atmosphere, thus enabling long-term cultivation over several days to weeks. Cell compatibility is tested for different culture periods.

Our bioreactors offer new perspectives for *in vitro* studies on mechanobiological processes in regenerating tissues. In addition, it represents a first step towards developing airway replacement tissues that may in the future provide new treatment options for cancer patients.

A Free-form Patterning Method Enabling Endothelialization under Dynamic Flow

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Surface structuring is a powerful tool to guide cell responses in both biomedical engineering and mechanobiological research. Rational engineering of the surface micro-topography at the luminal interface of ventricular assist devices provides an access point to support the survival of a living endothelium. This effect is particularly important under the challenging hemodynamic conditions created by the implant deployment and function. Such cell conditioning was shown to be done through various mechanotransduction pathways. Here, a novel patterning method, harnessing the condensation and evaporation of water droplets on a curing liquid elastomer, is developed to introduce arrays of microscale wells on the surface of a biocompatible silicon layer. The resulting topography support the in vitro generation of mature human endothelia and their maintenance under dynamic changes of flow direction or magnitude, greatly outperforming identical, but flat substrates. This topography is shown to interfere with flow-sensing related junctional marker VE-Cadherin, as well as the Wnt/β-catenin pathway in endothelial cells.

Chemo-Mechanical Triggering of Nucleo-Cytoskeletal Coupling using Fluidic Force Microscopy and Fluorescence Lifetime Imaging Microscopy

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Understanding the interactions between biochemical and mechanical cues in the cellular behaviors is a key for revealing numerous biological functions and development of major diseases. This necessitates creating tools enabling *in situ* monitoring and quantifying such small forces in single-cell levels while preserving cell viability and avoiding influences on the ongoing cellular events. Mechanosensory complexes at the cellular level are such a demanding situation in which the dynamic interconnectivity of the cytoskeleton, nuclear envelope, and nucleoskeleton is yet to be understood. Fluorescence lifetime imaging microscopy (FLIM) is advantageous for probing such molecular environments using fluorophores report the membrane tension changes through their fluorescence lifetime changes.

Fluidic force microscopy (FluidFM), a force-controlled micropipette configured on an atomic force microscope (AFM) enables to manipulate intact cells with simultaneous screening of molecular responses. FluidFM micro-manipulation of single cells includes but not limited to quantitative injection of impermeable molecules and extraction of biomarkers, transcriptomes, and other biomolecules into and from cytosol (or nucleus) of living cells. This provides noticeable evidence on the single cell dynamics and therefore numerous biological processes in a spatially defined fashion.

FluidFM combined with FLIM allows for mechano-chemical manipulation of intact cells along the parallel *in situ* mapping of cellular responses over single cells. Thanks to the versatility of FluidFM tips and gentleness of cantilever-membrane contact, cell viability is preserved and influences on the ongoing cellular events is minimized. Thereby, I will elaborate FluidFM combined with FLIM-based sensory systems to address the dynamic mechanosensory of essential components of the linker of nucleoskeleton and cytoskeleton (LINC) complex by *in situ* 3D stress mapping over the nuclear envelope.

YAP Knockout Suppresses Early Germ Layer Specification of Human iPSC-Derived Embryoid Bodies

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Induced pluripotent stem cells (iPSCs) form aggregates that recapitulate aspects of the self-organization in early embryogenesis. Within few days, the cells undergo a transition from epithelial-like structures to organized three-dimensional embryoid bodies (EBs) with upregulation of specific genes for endodermal, mesodermal, and ectodermal differentiation. The Yes-associated protein (YAP) is a downstream effector of the Hippo pathway and an essential mechanotransducer during early mouse embryo development. In this study, we further investigated the relevance of YAP for directed germ layer differentiation and self-organization in human EBs.

We used CRISPR/Cas9 technology to generate iPSC lines with homozygous *YAP* knockout (*YAP*^{-/-}). *YAP* deficiency hardly affected pluripotent state, as reflected by typical iPSC morphology, maintained expression of the pluripotency marker OCT-4, and a positive epigenetic Epi-Pluri-Score. Nevertheless, RNA-sequencing revealed significant effects on expression of various genes that are relevant for cellular differentiation, including *NODAL*, coding for an important signaling protein of the TGF-β pathway. Subsequently, wildtype and *YAP*^{-/-} iPSCs were differentiated towards endodermal, mesodermal, and ectodermal lineages using two-dimensional media-directed differentiation assays. Immunofluorescence revealed a similar expression of germ layer markers with only moderate impact of *YAP* deficiency on gene expression profiles. To gain further insight into the epigenetic sequel of *YAP* deficiency, we also investigated DNA methylation profiles before and after trilineage differentiation. The *YAP*^{-/-} cells revealed some epigenetic aberrations, but overall the directed lineage-specific differentiation potential seemed to be maintained upon treatment with induction media. In contrast, a complete loss of endodermal, mesodermal, and ectodermal differentiation was observed in three-dimensional EBs that were generated from *YAP*^{-/-} iPSCs – there were no subpopulations with a clear up-regulation of GATA6, Brachyury, and PAX6, respectively. A *YAP* rescue via lentiviral transfection partially recovered the phenotype that was generated by *YAP* deficiency. Gene expression and gene set enrichment analysis resulted in significant downregulation of germ layer development and embryogenesis related genes and pathways in *YAP*^{-/-} EBs. *YAP* deficiency mainly affected the TGF-β signaling pathway, which is involved in mesoderm and endoderm induction and embryo differentiation.

These results demonstrate that *YAP* plays an important role for mechanotransduction during early germ layer specification of iPSCs, particularly during self-organization and differentiation of EBs.

Optimization of lectin patterns for selective cancer cells capturing

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In the present study, we fabricated two-component lectin patterned substrates used to capture bladder normal and cancer cells. We used wheat germ agglutinin (WGA) and phytohemagglutinin-L (PHA-L) lectins. WGA and PHA-L were chosen based on transmigration and adhesion experiments.

For protein patterning microcontact printing (μ CP)-based approach has been used. After μ CP, the second protein was deposited by the drop-casting method. Fluorescence microscopy (FM) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) characterized the chemical composition and patterns homogeneity.

Although FM images indicated selectivity and good replication quality of the stamp pattern, the ToF-SIMS analysis revealed two issues. Firstly, during μ CP of lectins, silicone transfer occurs, and WGA-covered areas are contaminated. Secondly, adsorption of PHA-L results in the lectin adsorption not only at complementary patterns but also at WGA areas. Due to these drawbacks, other μ CP-based approaches have been used. All substrates were tested to verify the selectivity of protein deposition and silicone transfer issues.

The biological activity of dual-protein patterns, prepared by applying different μ CP-based approaches, has been tested using single-molecule force spectroscopy (SMFS). We have also examined cell sensitivity to silicone contamination by static adhesion experiments.

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4D force detection of cell adhesion and contractility combining FluidFM and confocal reference-free TFM

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Fluid Force Microscopy (FluidFM) combines a hollow microchannel cantilever with conventional AFM and has proven to be a promising tool in biological applications, especially in cell mechanics. (1) FluidFM and Atomic Force Microscopy (AFM) provide abundant force sensitivity in the perpendicular direction but lack information in the xy lateral direction. Confocal reference-free Traction force microscopy (cTFM) is a powerful tool for the quantification of cellular forces and allows the continuous high-resolution recording of force field without the need of a reference image. (2) By combining these two methods for the first time, a full time-resolved volumetric force detection (4D) is feasible, more specifically contractile force detection by cTFM and z interaction force detection by FluidFM. This opens opportunities in answering biological questions relative to cell mechanics with unprecedented spatial and temporal resolution. How cells interact with their surrounding is an essential topic because the mechanical machinery and cell signaling are fundamental to complex biological processes such as tissue development and crucial to biomaterials design and study of pathology. The generation of cellular forces is mainly based on the actomyosin apparatus and their transmission to the substrates is through integrin-based adhesions. (3) Although FluidFM is proficient in measuring the local elasticity and adhesion strength quantitatively, the broader microscopic traction force information at the basal side, where interaction with the substrate is established, is not available. In this frame, cTFM measuring the traction forces generated by cells provides an additional, all-round perspective.

By combining the main features of these two techniques, it is possible to decouple the cell adhesion and contractility during active manipulation of individual cells. To obtain quantitatively the adhesion force of single cells, an individual cell will be pulled from the substrate until detachment. This measurement is commonly referred as Single Cell Force Spectroscopy (SCFS). (4) However, the basal contractile forces during SCFS measurements have not yet been investigated.

We perform concurrently the SCFS and cTFM measurements of HeLa cells (control) and cells with disassembled actin cytoskeleton due to treatment with cytochalasin D. We show that the loss of contractility caused by the decomposed cytoskeleton does not affect the cell adhesion. Our results indicate that integrin based adhesion and actomyosin contractility can be temporally resolved. In conclusion, we demonstrated that the combination of two innovative techniques is opening up a wealth of possibilities to answer biological questions by bringing force sensing quantitatively into 4D.

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