

**"Development and Standardization of the CellDrum™: A
Biofunctionalized Ultra-Thin PDMS Membrane Sensor for
Advanced Cell Culture and Mechanobiological Testing"**

Inaugural dissertation
for the attainment of the title of doctor
in the Faculty of Mathematics and Natural Sciences
at the Heinrich Heine University Düsseldorf

presented by

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Düsseldorf, October 2025

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Date of the oral examination: 22.06.2026

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Summary

This work focuses on the optimization, characterization, and validation of the CellDrumTM technology. A CellDrum is a physical cell force sensor. It consists essentially of an ultra-thin, flexible PDMS membrane that seals one side of a cylindrical base body (applicator) in a water-tight manner. When filled with cell culture medium, the membrane bends slightly downward in relation to its diameter. This deflection can be measured. Cell and thin tissue layers can be cultivated on the membrane. They then form a composite membrane consisting of living cells, extracellular matrix and PDMS. The tensile forces that develop in the connected cell layer change the mechanical properties of this composite membrane and thus its deflection. The PDMS membrane itself remains unchanged. The ratio of the number of cells causing the tensile stress to the PDMS membrane thickness and its preload is decisive in determining the sensitivity of these cell force sensors. Considering the low strength of the cell forces to be measured, it becomes clear what a challenge the manufacture and preparation of such sensors represents. The CellDrum technology was developed for advanced mechanobiological testing. The study was divided into three distinct experimental phases that systematically build upon each other.

In Phase 1, the CellDrumTM manufacturing process was optimized to determine critical parameters such as membrane thickness, uniformity, and mechanical stability. Quality control measures, such as preliminary visual inspection, interferometric thickness measurement as well as detection of membrane deflection, were performed to increase reproducibility and consistency of manually manufactured CellDrumTM batches. The effect of manufacturing variables, such as ambient temperature, on membrane thickness was also investigated, highlighting the importance of environmental control during production.

Phase 2 was focused on validating the biological functionality of CellDrumTM membranes through so-called “wound healing” assays using skin fibroblasts derived from young and aged human donors. In this study, the term “wound healing” was defined as the capacity of cultured cell monolayers to migrate and proliferate across an artificially generated cell-free gap (designated as a “wound”), with the progressive reduction in gap area serving as a quantitative measure of cellular repair processes. Cultured cells attached to the inner surface of the CellDrumTM membrane were tested. In order to stimulate the wound healing process in addition to intrinsic cell migration efforts, the cells were subjected to controlled mechanical stimulation. “Wound healing” dynamics over time was monitored using fluorescence microscopy by capturing images at predetermined time points. The results revealed age-dependent (cell donor age) differences in “wound healing” behaviour. Mechanical stimulation seems to have improved “wound” closing in “younger” cells, but showed less favourable or even adverse effects in “older” cell samples, highlighting the sensitivity of the system in detecting cell donor age-related mechanobiological differences.

In Phase 3, the system was used to measure the contractile responses of differentiated primary human aortic smooth muscle cell (HAOSMC) and primary human aortic endothelial cell (HAOEC) co-cultures after exposure to test substances, namely noradrenaline and ionomycin. Cell identity and phenotype were confirmed by immunofluorescence staining. Measurements were carried out with the Cell Force Analyzer (CFA_{refer}) enabling measurements of contractile cell tension of cell cultures attached to the CellDrumTM membrane allowing the assessment of dose-dependent contractile responses. While HAOSMC monolayers exhibited clear,

measurable contractility, the co-culture data showed more complex, nonlinear patterns that required alternative representations such as Excel scatter plots. Ionomycin was used as a positive control and confirmed the functional contractility of the model.

Overall, this work demonstrates that the CellDrumTM platform may actually serve as a reproducible, flexible, and physiologically relevant tool for mechanobiology research, with potential future applications in tissue engineering, pharmacological testing, and regenerative medicine.

Zusammenfassung

Diese Arbeit konzentriert sich auf die Optimierung, Charakterisierung und Validierung der CellDrumTM-Technologie. Eine CellDrum ist ein physikalischer Zellkraftsensor. Sie besteht im Wesentlichen aus einer ultradünnen, flexiblen PDMS-Membran, die eine Seite eines zylindrischen Grundkörpers (Applikator) wasserdicht verschließt. Wenn sie mit Zellkulturmedium gefüllt ist, biegt sich die Membran im Verhältnis zu ihrem Durchmesser leicht nach unten. Diese Durchbiegung kann gemessen werden. Auf der Membran können Zellen und dünne Gewebeschichten kultiviert werden. Sie bilden dann eine Verbundmembran aus lebenden Zellen, extrazellulärer Matrix und PDMS. Die Zugkräfte, die in der verbundenen Zellschicht entstehen, verändern die mechanischen Eigenschaften dieser Verbundmembran und damit ihre Durchbiegung. Die PDMS-Membran selbst bleibt unverändert. Das Verhältnis der Anzahl der Zellen, die die Zugspannung verursachen, zur PDMS-Membrandicke und ihrer Vorspannung ist entscheidend für die Empfindlichkeit dieser Zellkraftsensoren. Angesichts der geringen Stärke der zu messenden Zellkräfte wird deutlich, welche Herausforderung die Herstellung und Vorbereitung solcher Sensoren darstellt. Die CellDrum-Technologie wurde für fortgeschrittene mechanobiologische Tests entwickelt. Die Studie wurde in drei verschiedene experimentelle Phasen unterteilt, die systematisch aufeinander aufbauen.

In Phase 1 wurde der Herstellungsprozess von CellDrumTM optimiert, um kritische Parameter wie Membrandicke, Gleichmäßigkeit und mechanische Stabilität zu bestimmen. Qualitätskontrollmaßnahmen wie eine vorläufige Sichtprüfung, interferometrische Dickenmessung sowie die Messung von Membranverformungen wurden durchgeführt, um die Reproduzierbarkeit und Konsistenz der manuell hergestellten CellDrumTM-Chargen zu verbessern. Der Einfluss von Herstellungsvariablen wie der Umgebungstemperatur auf die Membrandicke wurde ebenfalls untersucht, wodurch die Bedeutung der Kontrolle der Umgebungsbedingungen während der Produktion deutlich wurde.

Phase 2 konzentrierte sich auf die Validierung der biologischen Funktionalität von CellDrumTM-Membranen durch sogenannte „Wundheilungs“-Assays unter Verwendung von Hautfibroblasten, die von jungen und älteren menschlichen Spendern stammen. In dieser Studie wurde der Begriff „Wundheilung“ als die Fähigkeit von kultivierten Zellmonolayern definiert, über eine künstlich erzeugte zellfreie Lücke (als „Wunde“ bezeichnet) zu wandern und sich zu vermehren, wobei die fortschreitende Verringerung der Lückenfläche als quantitatives Maß für zelluläre Reparaturprozesse diente. Es wurden kultivierte Zellen getestet, die an der Innenfläche der CellDrumTM-Membran hafteten. Um den Wundheilungsprozess zusätzlich zu den intrinsischen Zellmigrationsbemühungen zu stimulieren, wurden die Zellen einer kontrollierten mechanischen Stimulation unterzogen. Die Dynamik der „Wundheilung“ im Zeitverlauf wurde mittels Fluoreszenzmikroskopie durch Aufnahmen zu vorab festgelegten Zeitpunkten überwacht. Die Ergebnisse zeigten altersabhängige (Alter des Zellspenders) Unterschiede im „Wundheilungsverhalten“. Die mechanische Stimulation scheint die „Wundheilung“ in „jüngeren“ Zellen verbessert zu haben, zeigte jedoch weniger günstige oder sogar nachteilige Auswirkungen in „älteren“ Zellproben, was die Empfindlichkeit des Systems bei der Erkennung von altersbedingten mechanobiologischen Unterschieden der Zellspender unterstreicht.

In Phase 3 wurde das System verwendet, um die kontraktilen Reaktionen von differenzierten primären menschlichen Aortenglattmuskulzellen (HAOSMC) und primären menschlichen Aortenendothelzellen (HAOEC) in Co-Kulturen nach Einwirkung der Testsubstanzen Noradrenalin und Ionomycin zu messen. Die Zellidentität und der Phänotyp wurden durch Immunfluoreszenzfärbung bestätigt. Die Messungen wurden mit dem Cell Force Analyzer (CFA_{refer}) durchgeführt, der Messungen der kontraktilen Zellspannung von Zellkulturen ermöglicht, die an der CellDrumTM-Membran haften, wodurch die Bewertung dosisabhängiger kontraktiler Reaktionen möglich ist. Während HAOSMC-Monolayer eine klare, messbare Kontraktilität aufwiesen, zeigten die Co-Kultur-Daten komplexere, nichtlineare Muster, die alternative Darstellungen wie Excel-Streudiagramme erforderten. Ionomycin wurde als Positivkontrolle verwendet und bestätigte die funktionelle Kontraktilität des Modells.

Insgesamt zeigt diese Arbeit, dass die CellDrumTM-Plattform tatsächlich als reproduzierbares, flexibles und physiologisch relevantes Werkzeug für die mechanobiologische Forschung dienen kann, mit potenziellen zukünftigen Anwendungen in den Bereichen Gewebetechnik, pharmakologische Tests und regenerative Medizin.

1. Introduction

The mechanical environment fundamentally regulates cellular behaviour, influencing processes from embryonic development to cancer metastasis and cardiovascular disease. Cells continuously sense and respond to mechanical forces through mechanotransduction pathways, making cellular mechanobiology critical for understanding physiological and pathophysiological processes^{1,2}. Traditional cell culture systems use rigid substrates that fail to replicate the dynamic mechanical environments cells experience *in vivo*. While biological tissues exhibit elastic moduli ranging from hundreds of pascals (Brain- Pa) to mega-gigapascals (Bone), conventional culture dishes (Polystyrene ~ GPa) present substrates much stiffer than physiological conditions³⁻⁵. This mechanical imbalance significantly alters cell behaviour, gene expression, and drug responses, limiting the relevance of *in vitro* findings^{3,6-8}.

Aside from investigating various substrate materials and their effects on cellular behaviour, researchers have increasingly incorporated active mechanical stimulation into cell culture studies to better understand and replicate these mechanotransduction processes. Various approaches have been developed to apply controlled mechanical forces to cultured cells, which help bridge the gap between traditional static culture conditions and the dynamic mechanical environment of living tissues. However, existing approaches to incorporate mechanical stimulation into culture conditions via hydrogel substrates⁹, stretching devices¹⁰⁻¹², fluid flow chambers¹³, and compression systems¹⁴ suffer from limitations such as complex setup requirements, limited throughput, difficulties in real-time monitoring, or inability to precisely control multiple mechanical as well as physical parameters, like experimental temperature precision^{15,16} simultaneously. Many of these systems lack the standardization quality necessary for reliable pharmaceutical routine screening applications.

The CellDrumTM technology addresses these challenges through an ultra-thin, flexible PDMS membrane serving as a cell culture substrate with customizable mechanical properties. Developed and patented in the early 2000s, the system consists of a flexible membrane for cell seeding, strongly adhered to supportive applicator compatible with cell culture and analytical techniques¹⁷. The membrane's exceptional thinness and flexibility allow it to mimic *in-vivo* biomechanical conditions considerably more closely (~20-40 kPa) than conventional substrates. This provides a biomechanical environment that results in more physiologically relevant cellular responses. The underlying principle of this technology relies on membrane deflection mechanics. A PDMS membrane which deflects slightly under the weight of the cell culture medium is lifted due to cellular contractile tension when cells are cultivated and attached on its surface, thus performing mechanical work¹⁸. Even when biochemically or pharmacologically active substances alter the contraction of the cells, their deflection changes, which can be measured and used to calculate the mechanical tension of the cell layer^{19,20}.

A newly available instrument called CFA_{refer} has recently been brought to market that allows routine measurement of cell force based on CellDrumTM technology^{21,22}. The CFA_{refer} enables real-time, non-invasive monitoring of the mechanical tension of the cultured cell layers on the CellDrumTM membrane as well as of induced cell tension changes²¹.

Real-time monitoring of cell mechanical tension after controlled stimulation enables assessment of drug action dynamics that are crucial for optimizing therapeutic efficacy while simultaneously minimizing adverse effects.

While the CFA_{refer} focuses on the mechanical tension measurement capabilities, it can also provide mechanical stimulation, though this represents a secondary function compared to its main role as a monitoring system. The instrument, known as the PulSElect system, is used for the mechanical stimulation of adherent cells of any kind cultivated on CellDrum membranes. It is based on the reversal of the principle of cell force measurement with the CFA_{refer}. External pressure raises and lowers the membrane according to predefined stimulation parameters, frequency, amplitude and pulse waveform. This pressure induced movement of the membrane results in a biaxial mechanical stimulation of the cell layer adhered to it. It represents an alternative to other stretching protocols and, due to its rotationally symmetrical, biaxial stimulation, provides a much better approximation of the physiological, mechanical in vivo environment of the cells. Precise control of the stimulation parameters further supports and contributes to this ^{23,24}. PulSElect was used so far to introduce mechanical stimulation and detect changes in cellular biomechanical strength, stress fibre orientation, protein expression patterns after stimulation as well as gene expression studies ^{25–28}.

CellDrumTM technology is used both directly as a cell force analyser (CFA_{refer}) and indirectly as a mechanical cell stimulator (PulSElect) for all adhesion-dependent cells, going far beyond simple monolayer cell studies or specific cell types ^{18,19,29}. In contrary, it is compatible with various cell types like primary cells and even co-cultures ¹⁹. Its ability to also support 3D culture as well as co-culture configurations opens possibilities for studying semi-tissue-level responses and cell-cell interactions under physiologically relevant conditions. It also enables modelling of complex pathological processes like wound healing, vascular remodelling, and tissue fibrosis. A decisive key feature of the CellDrumTM production technology is its standardized manufacturing protocols and quality control measures. These are essential for ensuring highly reproducible data acquisition with the CFA_{refer}, and for making results comparable across different laboratories. This alignment with preclinical testing requirements helps to overcome a significant challenge in the field. Additionally, the properties of the PDMS membrane make the platform compatible with screening approaches, enabling the evaluation of various chemical compounds under physiologically relevant mechanical conditions. This further provides unique insights into drug behaviour that can complement existing testing methods.

From a pharmaceutical perspective, it is essential that this technology allows for the development of standardized protocols for testing substances, known as Standard Operating Procedures (SOPs). These are vital to ensure that substances are always tested in the same way, which makes it easy to test different cell types in a standardized manner. For example, SMCs from the aorta can be used instead of SMCs from the coronary arteries to investigate their location specific effects, since VSMCs display diversity in function and phenotype depending on their location within the arterial tree, their embryologic origin, and their organ-dependent microenvironment ³⁰. The previously mentioned standardized features are not easily realized with animal organ based experiments. In fact, relying on animal organ models present significant challenges and ethical concerns ³¹. Beyond the ethical problems associated with

animal organ explants, which require the sacrifice of laboratory animals, there are substantial physiological differences between animal and human drug metabolism that can make it difficult to translate preclinical findings to human applications. Moreover, the CellDrumTM technology with CFA_{refer} enables targeted investigation of the contributions and interactions of different cell types in co-cultures, which becomes experimentally feasible in controlled in vitro systems. Additionally, this approach offers significant cost reduction by eliminating expensive animal organ preparations, while also reducing the high variability of results typically observed with animal organ experiments. These features, along with the universal use of adhesion-dependent cells, make it possible to consider replacing traditional organ experiments, such as vascular rings³² or the Langendorff heart³³.

In conclusion, the CellDrumTM technology overcomes some of the current limitations in mechanobiology research tools and establishes a foundation for very broad applications. The system's potential to contribute to alternative testing approaches could support more informed decision-making in preclinical studies, potentially enabling more targeted use of animal organ models and even minimize their usage while improving pharmaceutical development processes. Beyond its immediate applications in mechanobiology and pharmacology, this technology holds promise for advancements in also regenerative medicine, toxicology, fundamental cell biology research, and material science, even in contexts not directly involving cellular systems.

1.1 Mechanobiology

Mechanobiology is a field that combines biology, engineering, and physics to study how mechanical forces influence biological processes from the molecular to the tissue level. The core idea is that these forces can directly alter cellular functions like cell shape, movement, growth, and even gene expression. This process, known as mechanotransduction, involves cellular components such as the cytoskeleton, cell membrane, and extracellular matrix (ECM) working together to turn mechanical signals into biochemical responses^{4,34}.

The role of mechanobiology is critical in molecular and cellular research, as it provides insights into the fundamental processes underlying various physiological and pathological conditions. For instance recent studies in this field have demonstrated how endothelial cells respond to shear stress to regulate blood vessel formation and function, osteocytes sense mechanical loading to preserve bone integrity, and cancer cells utilize the mechanical properties of the tumour microenvironment to promote metastasis^{35–37}. These insights hold significant implications for developing therapeutic strategies for diseases such as cancer, cardiovascular diseases, and osteoporosis. A better understanding of these pathways could also improve the design of biomaterials and tissue engineering, creating environments that mimic natural mechanical cues to achieve desired cellular responses.

Techniques such as atomic force microscopy (AFM)³⁸, traction force microscopy (TFM)³⁹, and microfluidics⁴⁰ have enabled manipulation and measurement of mechanical forces at cellular and subcellular levels. Research is increasingly focused on unravelling the molecular mechanisms regarding mechanosensing and mechanotransduction, identifying key proteins and pathways involved, as well as how diseases effect these processes^{41,42}.

1.1.1 CellDrum™ Technology and PDMS

The CellDrum™ was invented by Prof. Dr. rer.nat. habil. Gerhard M. Artmann in the early 2000s and has been further developed at the University of Applied Sciences Aachen, Center of Competence in Bioengineering. The CellDrum™ applicator, is constructed from a Polyetheretherketone (PEEK) cylinder, sealed at its lower side by a membrane composed of polydimethylsiloxane (PDMS). The CellDrum™ cylinders used in this study have a diameter of 16 mm, a height of 8 mm, and provide a total inner membrane surface area of 2.01 cm² for cell attachment.

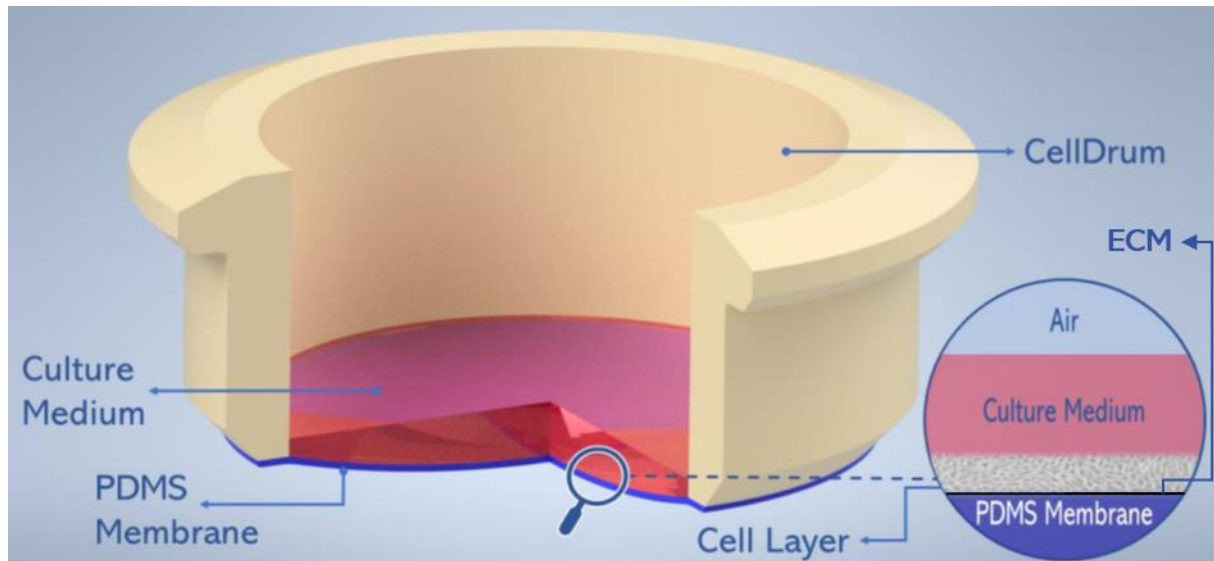


Figure 1: Schematic representation of the CellDrum™ system

PDMS is a silicon-based elastomer often utilized in the field of cellular biomechanics due to its unique combination of properties. It is a polymer consisting of $\text{-OSi(CH}_3\text{)}_2\text{-}$ monomers in which silicon and oxygen form the main chain alternately, while two methyl groups are bound to the silicon ⁴³. Its optical transparency, mechanical flexibility, biocompatibility, and gas permeability make this material ideal for replicating mechanical environments and studying how cells respond to physical stimuli, enabling progress in areas such as tissue engineering, mechanobiology, and disease modelling ^{43–45}. A key advantage is that PDMS's stiffness is highly tunable, typically ranging from 1–4 MPa but capable of falling into the lower kPa range by adjusting the thickness and/or mixing ratios ^{46,47}.

A big disadvantage of PDMS is its inherent hydrophobicity, primarily attributed to the abundance of non-polar methyl groups on its surface ^{44,45}. This hydrophobic characteristic greatly reduces the suitability of this material for direct use in cell culture applications, as it limits initial protein adsorption and prevents stable cell adhesion which is essential for CellDrum™ technology. Cells generally require an underlying layer of ECM proteins, such as fibronectin, collagen or laminin, to attach, spread, and retain biological function ⁴⁸. However, PDMS surfaces fail to maintain stable binding of these proteins over time leading to cellular apoptosis ^{44,49}. In addition, the hydrophobicity of PDMS can lead to the nonspecific adsorption or absorption of small molecules and soluble factors present in the culture medium or secreted by cells ⁵⁰. These molecules can interfere with cellular communication, affecting cell behaviour,

viability, and experimental outcomes. In addressing these challenges, a variety of surface modification techniques have been developed to increase PDMS hydrophilicity, such as oxygen plasma treatment, ultraviolet (UV)/ozone exposure, or covalent grafting of hydrophilic and bioactive coatings, to improve surface wettability, stabilize protein attachment, and facilitate reliable cell adhesion and proliferation^{45,48–51}.

Several chemical and physical modification strategies enhance PDMS bioactivity and cellular compatibility^{52,53}. Oxygen plasma and UV/ozone treatments temporarily increase surface hydrophilicity by introducing polar functional groups, though both suffer from hydrophobic recovery within hours to days^{51,52}. More stable approaches include chemical grafting, where hydrophilic molecules like polyethylene glycol (PEG) are covalently bonded to surfaces, significantly enhancing long-term protein retention and cell adhesion. Sol-gel coatings deposit silica-based layers onto PDMS, offering improved wettability and mechanical stability for dynamic culture environments. Additionally, polymer blending or surface layering with hydrophilic polymers provides permanent surface property alterations. While each method offers distinct advantages, they vary in complexity and long-term stability, highlighting the importance of careful selection based on specific experimental needs^{45,51,52}.

Common PDMS membrane production methods include spin-coating, casting, and soft lithography^{54–56}. Spin-coating dispenses liquid PDMS mixture onto substrates and spreads it into thin, uniform layers through high-speed rotation⁵⁴. Casting involves spreading the PDMS prepolymer using a casting knife to achieve a desired thickness⁵⁶. Soft lithography utilizes mould-based casting techniques, enabling precise thickness control and surface microstructure incorporation⁵⁵. However, none are suitable for consistently producing membranes thinner than 10 μm with sufficient surface area and mechanical properties for cellular-scale stress sensing. Applications measuring mechanical forces on monolayers only a few micrometers thick demand high standards like uniform membrane adhesion, wrinkle prevention, and consistent mechanical preloading across surfaces⁴⁷. These criteria are essential for reproducible mechanical tension measurements in cell biomechanics.

A specific method enabling the fabrication of ultra-thin PDMS membranes was significantly further developed in this thesis, in which the PDMS elastomer was cured on the surface of water. This approach allowed the elastomer to spread evenly across the target area, resulting in significantly thinner membranes compared to those produced by conventional PDMS fabrication techniques. Although a similar membrane fabrication method, referred to as the "floating-on-water" (FoW) process⁵⁷, was reported by another research group in 2019, this approach has been invented and independently employed in our laboratory since the early 2000s^{17,18}.

PDMS membranes are commonly used to study mechanical properties of single cells or cell layers in vitro through compressive or longitudinal tensile loads. Various measurement techniques allow calculation of cell tensile forces, deformation and stiffness by modifying commercially available instruments by research groups^{10–12,58}. These companies offer mechanical traction applications with uniaxial and biaxial stretching options, plus compression systems mimicking complex physiological and biomechanical nature of cell and tissue mechanics¹⁴. Cell seeding areas in membrane arrays vary from 1 to 10 cm^2 with membrane thicknesses of 100–500 μm ^{10,12}. These membranes are available "untreated" or "functionalized"

for improved cell adhesion, with stiffness modulus ranges of 1-60 kPa⁵⁸. Though few disposable membranes are commercially offered for these devices, some systems still required researchers to produce their own membranes, leaving room for membrane production optimization and post-production quality control. Traditionally, tensile stress experiments on cell monolayers predominantly utilize uniaxial stretching methods due to their relative simplicity and ease of implementation. However, uniaxial loading does not truly mimic the complex mechanical environments that cells experience in-vivo, where forces are often multidirectional. Biaxial mechanical stimulation offers a more physiologically relevant approach by applying stress along two perpendicular axes, closely mimicking the multidirectional strains encountered by cells within native tissues. Studies have demonstrated that biaxial loading can lead to different cellular responses compared to uniaxial loading, affecting aspects such as cytoskeletal organization, gene expression, and ECM production. For instance, Meiner et al. determined that chondrocytes subjected to biaxial mechanical loading exhibit enhanced accumulation of cartilage-specific ECM components, suggesting improved tissue functionality⁵⁹.

The CellDrumTM membrane has thickness ranging between 3 to 4 μm , offering optimal balance between flexibility and durability while providing a soft surface for cell growth. However, PDMS membrane thickness can be fine-tuned between 2-10 μm to accommodate specific experimental requirements, allowing customization based on mechanical properties needed for different cellular studies. This adjustability ensures membrane mechanical environments can be tailored to optimize growth and experimental conditions for various cell types and research applications. Furthermore, its ability to subject the overlying cell monolayer to biaxial cyclic mechanical tension opens the door to investigating the effects of varying mechanical environments, establishing this technology as a robust platform for studying cellular biomechanics and mechanotransduction⁴⁷.

1.1.2 Cellular Forces

Many cellular processes are modulated by external and internal mechanical forces that cells experience^{1,60}. Ouyang et al. demonstrated that traction forces exerted by cells on ECM can propagate over considerable distances, enabling distant mechanical communication that guides alignment, migration, and self-assembly within three-dimensional environments⁶¹. This highlights the critical role of matrix-mediated force transmission in collective cellular behaviours and mechanotransduction^{61,62}.

Building on this, cells also process signals from multiple sources. They use membrane-bound receptors to convert biochemical cues from growth factors, hormones, and ECM components into mechanical responses. At the same time, they interpret mechanical cues from their surroundings through cytoskeletal contractility and cell-ECM adhesion. These continuous mechanical interactions regulate cellular activities like differentiation, proliferation, migration, and apoptosis⁶³⁻⁶⁵. Various cell types are highly mechanosensitive. A prime example is fibroblasts, which generate significant traction forces to produce and remodel the ECM, guiding tissue structure during processes like wound healing and fibrosis. Similarly, osteoblasts and vascular endothelial cells are continuously subjected to mechanical stimuli, while muscle cells

specialize in converting biochemical or electrical signals into mechanical forces to enable cellular contraction and movement ^{1,66}.

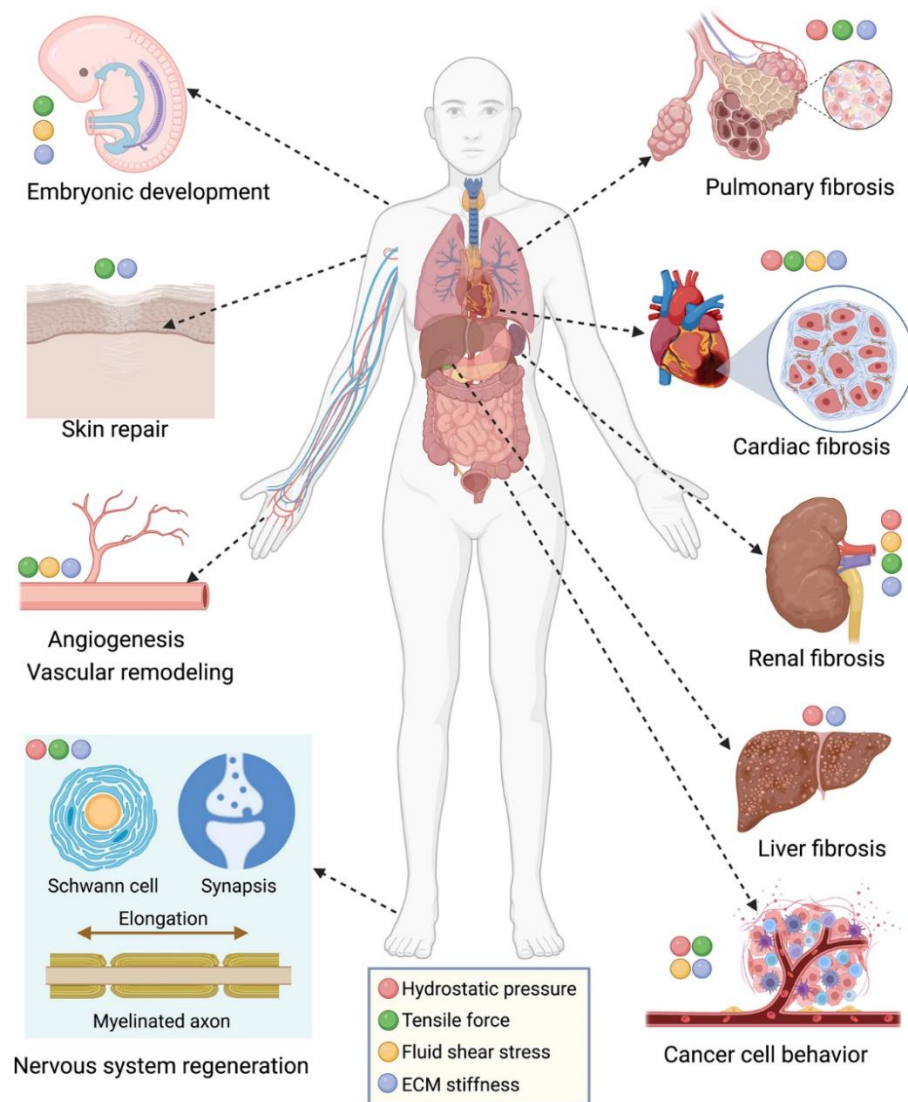


Figure 2: Overview of mechanical cue-driven pathophysiological mechanisms ¹

Cells continuously interpret and respond to diverse physical cues in living organisms including hydrostatic pressure, fluid shear stress, tensile forces, ECM stiffness, tissue elasticity, and interstitial fluid viscosity ¹. They rely on these cues to modulate key functions through mechanotransduction, converting mechanical stimuli into biochemical responses ^{42,67}. For example, endothelial cells respond to flow patterns (laminar, pulsatile, or disturbed) by altering behaviour to maintain vascular homeostasis or, under pathological conditions, contributing to diseases like atherosclerosis ^{2,68–70}.

Fibroblasts sense ECM stiffness and generate contractile forces to close tissue gaps and remodel matrix architecture during wound healing ^{29,71,72}. Muscle cells convert biochemical or electrical signals into mechanical contraction, regulated by cytoskeletal tension and force transmission ⁷³. Additionally, cancer cells use altered tissue mechanics such as increased matrix stiffness and solid stress to promote metastasis ⁷⁴.

To investigate these interactions under controlled conditions, researchers increasingly utilize in vitro systems designed to replicate in vivo mechanical environments. Several advanced techniques have been developed to measure the forces that cells generate and respond to within their microenvironment. Traction force microscopy (TFM) and micropillar arrays quantify forces that adherent cells exert on substrates, providing insights into cellular contractility and mechanotransduction^{62,75,76}. AFM and optical/magnetic tweezers are used to measure local mechanical properties and apply forces with high resolution. Microfabricated devices and deformable substrates are used to study cellular forces under dynamic conditions, such as cyclic or constant loads. Bioreactors and mechanical stimulation platforms apply defined loads like strain, shear stress, or pressure to cultured cells, providing tools for research in development, pathology, and regenerative medicine^{10,12,60}. However, many of these systems have limitations, including being invasive, focused on single-cell measurements, or relying on specialized, expensive equipment. As such, developing alternative methods for cell force analysis is an ongoing area of research, with the goal of creating accessible and more physiologically relevant measurements at the cell layer or tissue level.

1.1.3 Mechanotransduction

Mechanotransduction represents the fundamental process by which cells convert mechanical stimuli into biochemical responses, serving as the molecular bridge between physical forces and cellular behaviour^{1,65,77}.

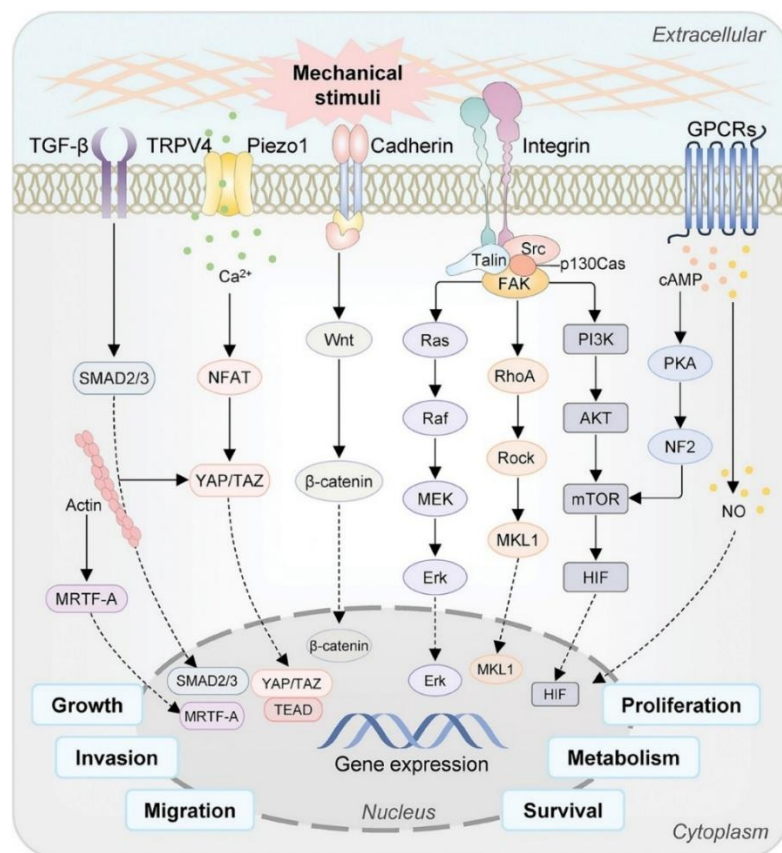


Figure 3: Overview of key mechanotransduction signalling pathways⁷⁷.

Primary mechanosensors (Figure 3) responsible for detecting external forces are concentrated at focal adhesions, dynamic protein complexes that physically connect ECM to cellular cytoskeleton through integrin receptors. These integrin-based adhesion sites act as the primary interface where mechanical forces are transmitted between ECM and intracellular cytoskeleton, establishing mechanical reciprocity that enables force sensing and transduction⁷⁸. Mechanosensitive ion channels, particularly the PIEZO family, complement integrin-mediated mechanosensing by providing rapid cellular responses to mechanical stimuli through calcium influx and membrane potential changes^{1,77,79}. Recent evidence demonstrates regulatory crosstalk between focal adhesions and PIEZO1 channels, highlighting their interconnection⁷⁹.

At the molecular level, mechanotransduction involves signalling pathways that translate mechanical inputs into cellular responses (Figure 3). The YAP/TAZ pathway serves as a central mechanotransduction hub, with these transcriptional regulators being regulated by mechanical stimuli through integrin-dependent signalling cascades^{1,80}. The Rho/ROCK signalling pathway plays crucial roles in cytoskeletal remodelling and contractility in response to mechanical forces, while focal adhesion kinase (FAK) signalling coordinates force-dependent adhesion dynamics^{78,81}. These pathways work together to regulate cellular processes such as proliferation, differentiation, and migration in response to mechanical properties of the cellular environment.

1.2 Cellular Biomechanics of Key Mechanosensitive Cell Types

Cellular biomechanics involves the study of mechanotransduction pathways, cytoskeletal dynamics, and the mechanical properties of cells and tissues that enable cells to function *in-vivo*^{34,77}. Among the diverse cell types, certain populations demonstrate particularly pronounced mechanosensitive characteristics and play pivotal roles in maintaining mechanical tissue homeostasis through complex molecular machinery that allows them to sense mechanical stimuli, convert these physical signals into biochemical responses, and generate contractile forces that contribute to tissue function, remodelling and wound healing^{66,82,83}.

Three cell types we chose as exemplary models for studying cellular biomechanics due to their physiological significance within the dermal microenvironment were: dermal fibroblasts^{72,83}, endothelial cells^{70,84} and smooth muscle cells⁸⁵. While cardiomyocytes and skeletal muscle cells are well-established as the most prominent force-generating cell types, our focus centers on the mechanobiology within dermal tissue architecture, where these three selected cell populations collectively contribute to skin homeostasis, vascular function, and wound repair. Cardiomyocytes have been investigated in a separate study within our laboratory, while skeletal muscle cells, though mechanically significant, introduce additional complexity that falls outside the defined scope of dermal tissue mechanics examined in this work²⁰. Each of these cell types exhibits unique mechanotransduction mechanisms and contributes differently to tissue mechanics, making them valuable subjects for both individual characterization and co-culture studies aimed at understanding cell-cell interactions at a mechanical level².

1.2.1 Dermal Fibroblasts

Fibroblasts belong to the connective tissue cell family and are responsible for producing collagen-rich ECM components, including various collagen types, proteoglycans, laminin, fibronectin, and tenascin^{27,72,86}. Despite being the least specialized cells in their family, fibroblasts possess high differentiation potential, adapting to various tissue environments. Regional differences affect secreted protein volumes for homeostasis, such as collagen and the ratio between types I and III^{87–89}.

The biomechanical properties of skin, including its tensile strength, elasticity, and viscoelastic behaviour, are directly dependent on the organization and composition of this matrix produced by the fibroblasts^{72,87,88}. Under tensile stress, fibroblasts align cytoskeletal elements along force direction and increase collagen synthesis through integrin-based focal adhesion complexes containing mechanosensitive proteins like FAK, talin, vinculin, and paxillin^{27,83,90}. Contractile forces from the acto-myosin apparatus contribute to matrix remodelling and collagen fiber alignment, while integrin mediated signalling enables both mechanotransduction and tissue damage detection to trigger repair responses^{66,78,91}.

Changes relating to aging also significantly impact fibroblast biomechanical function, leading to alterations in collagen structure of the tissue and mechanical properties^{92–94}. Aging fibroblasts show reduced mechanical sensitivity, decreased collagen production, and altered crosslinking patterns, resulting in characteristic loss of skin elasticity and mechanical integrity^{94–96}. These changes impair wound healing and increase susceptibility to injury, highlighting fibroblasts importance in maintaining skin integrity.

1.2.2 Endothelial Cells

The dermal vasculature, composed primarily of endothelial cells, plays a crucial role in skin homeostasis and represents another key component in skin tissue biomechanics. These cells form the inner lining of capillaries, arterioles, and venules, experiencing various mechanical forces including fluid shear stress from blood flow, circumferential stretch from pulsatile pressure, and transmural pressure differences^{69,70}.

These cells possess complex mechanosensory pathways including the glycocalyx, ion-channels, integrin-based focal adhesions, and cell-cell junctions. The endothelial glycocalyx serves as the main mechanotransducer for fluid shear stress, initiating downstream signalling cascades essential for vascular homeostasis, angiogenesis regulation, and inflammatory response coordination. Shear stress promotes cellular alignment in the flow direction and stimulates vasoactive molecule production such as nitric oxide, which regulates vascular tone and permeability through endothelial nitric oxide synthase (eNOS) activation^{35,70,84,97}.

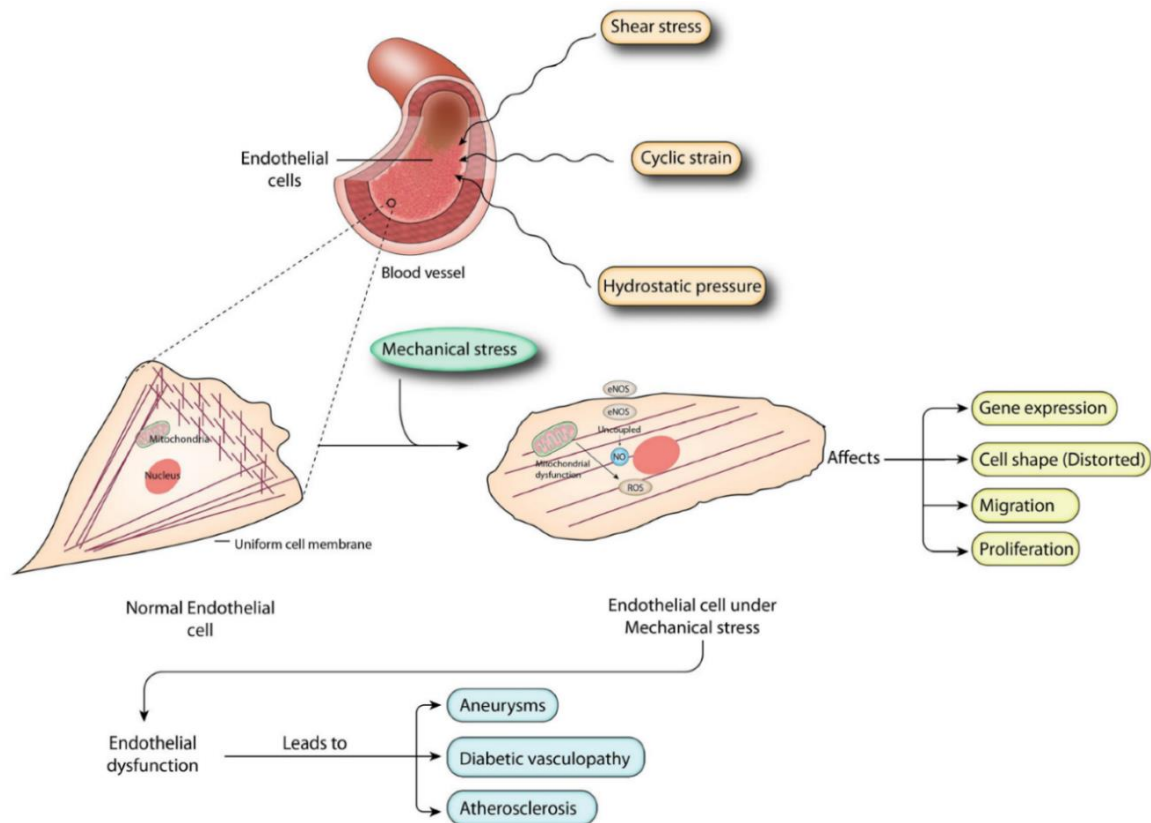


Figure 4: Mechanical Forces in Vascular Endothelium: From eNOS Activation to Pathological Outcomes ⁸⁴

The eNOS response occurs in two phases: acute activation (seconds to minutes) involving post-translational modifications, and chronic responses (hours to days) including transcriptional upregulation, allowing both immediate vasodilatory responses and long-term adaptation to changing hemodynamic conditions. Additionally, physiological shear stress strengthens intercellular junctions by promoting assembly of junctional proteins like VE-cadherin, reducing vascular permeability and maintaining endothelial barrier function ^{97,98}. Through these pathways, endothelial cells function as critical mediators in vascular mechanobiology, translating hemodynamic forces into tissue-level responses that maintain dermal homeostasis.

1.2.3 Vascular Smooth Muscle Cells

Vascular smooth muscle cells (VSMCs) are found within blood vessel walls throughout the body, including within dermal blood vessels of the skin. These cells contain contractile apparatus composed of actin and myosin filaments, enabling sustained contractile forces essential for vascular regulation ⁸⁵. VSMCs are subjected to circumferential stretch from pulsatile pressure, axial stretch from vessel elongation, and transmural pressure differences, with their mechanical properties regulated through mechanosensitive ion channels, integrin-based focal adhesions, and the contractile apparatus itself ^{73,78,99}.

VSMCs in dermal vessels regulate blood flow and pressure through vasoconstriction and vasodilation modulated by mechanical and chemical stimuli, including wall tension, pressure changes, and vasoactive substances such as noradrenaline, endothelin-1, angiotensin II, and nitric oxide ^{85,100–102}. Mechanical forces trigger stretch-activated channels and TRP family channels, causing calcium influx that activates contractile machinery through calcium-

calmodulin interactions, while L-type voltage-gated channels amplify responses for sustained contraction^{19,99}.

Alterations in mechanotransduction can lead to pathological conditions, as chronic mechanical stress causes enhanced myogenic responses and VSMC hypertrophy, while age-related arterial stiffening reduces normal mechanical stimuli and impairs vascular reactivity⁹⁹. Understanding SMC mechanobiology is crucial for developing therapeutic approaches, as mechanical forces influence SMC differentiation, proliferation, and functional behaviour.

1.3 Cell Assays

1.3.1 Wound Healing

Wound healing represents one of the most complex and highly orchestrated biological processes, involving the interaction of multiple cell types, signalling pathways, and ECM components to restore tissue barrier function following injury. The healing response involves a cascade of cellular and molecular events categorized into three overlapping phases: inflammatory, proliferative, and maturation/remodelling phases, each characterized by specific cellular activities and ECM changes¹⁰³.

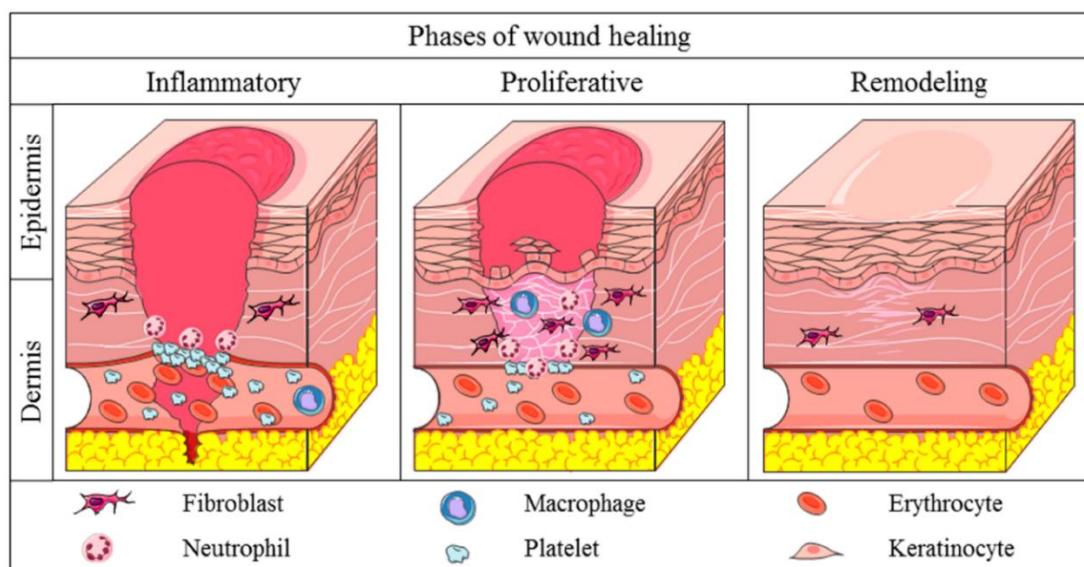


Figure 5: Three stages of wound healing and the corresponding helper cells¹⁰³

The inflammatory response is initiated by damage-associated molecular patterns (DAMPs) that activate pattern recognition receptors, leading to pro-inflammatory cytokine production including IL-1 β , TNF- α , and IL-6¹⁰⁴. Neutrophils perform pathogen elimination, followed by macrophage recruitment that transitions from M1 pro-inflammatory to M2 anti-inflammatory phenotypes driven by IL-4, -10 and -13, with M2 macrophages secreting growth factors that promote tissue repair^{83,103,104}.

The proliferative phase involves angiogenesis, granulation tissue formation, and re-epithelialization. Fibroblasts migrate into the wound area and become activated through TGF- β 1 signalling, proliferating and synthesizing collagen III, fibronectin, and other matrix proteins^{71,72,87,88}. Angiogenesis occurs via growth factors including VEGF and bFGF, while re-

epithelialization involves keratinocyte migration supported by fibroblast-produced keratinocyte growth factor^{83,104}.

During maturation/remodelling, fibroblasts differentiate into myofibroblasts under mechanical tension and sustained TGF- β signalling, characterized by α -SMA upregulation and contractile capabilities that enable wound contraction and increased collagen I production^{66,72,91}. Mechanical forces enhance healing through mechanotransduction pathways, with fibroblasts sensing forces through integrin-mediated focal adhesions and mechanosensitive ion-channels^{78,83,105}. Mechanical tension promotes myofibroblast differentiation and activates latent TGF- β 1, while cyclic stimulation upregulates type III collagen expression, supporting enhanced wound healing as demonstrated by Carver et al.^{83,90}. Successful ECM remodelling depends on controlled degradation through matrix metalloproteinases (MMPs) regulated by TIMPs, with gradual collagen III replacement by collagen I and fiber reorganization along tension lines increasing tensile strength^{87,90,91}. However, wound tensile strength rarely achieves unwounded skin levels due to matrix organization alterations, with abnormal collagen deposition leading to fibrous scars lacking resident dermal cells and having disorganized elastic fiber networks^{83,104}.

1.3.2 In Vitro Wound Healing Models

Wound assays are commonly used to test the effects of pharmaceutical and cosmetics ingredients, growth factors, chemokines, and cytokines on migration and proliferation in cell monolayers. The most used in-vitro method is the "scratch assay," in which a cultured cell monolayer is purposely damaged via water jets, cell scrapers, pipette tips, lasers, heated stamps, or electric currents^{106,107}.

Water/pressure jet systems utilize high pressure fluid streams but can cause cellular stress to adjacent cells and drive debris into the wound area. Alternatively, cell scrapers and pipette tips represent the most accessible methods, with P200 tips creating ~550 micrometre wounds and P1000 tips producing ~850 micrometre gaps, though wound quality depends on operator technique and can damage underlying matrix coatings, especially on softer substrates like silicone membranes¹⁰⁸.

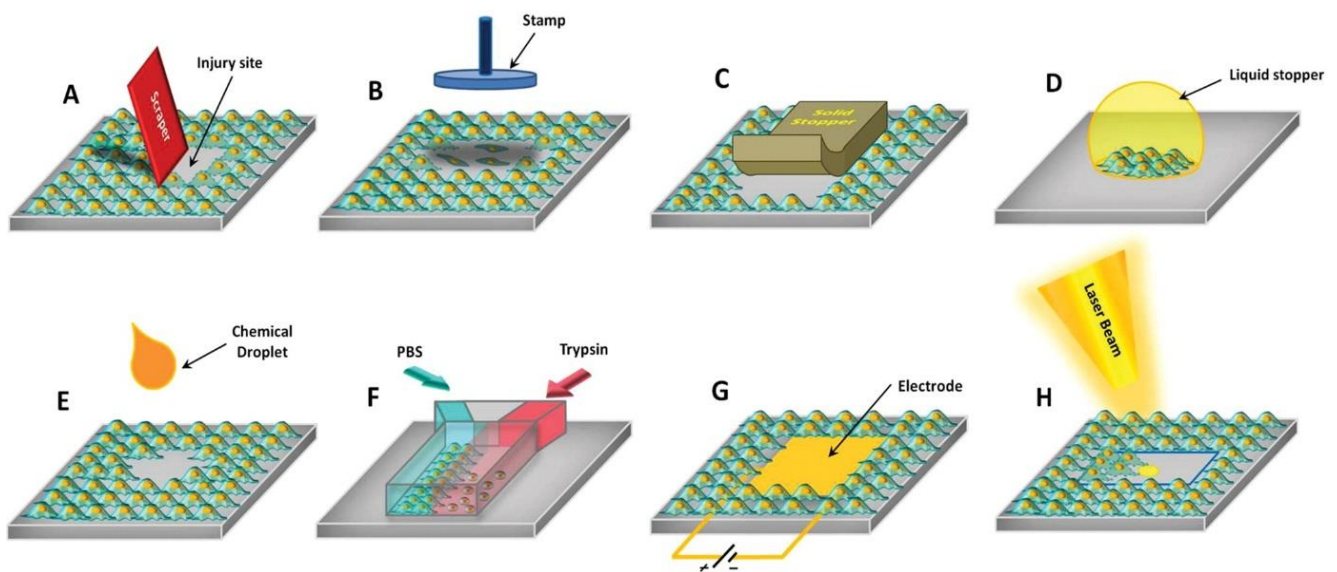


Figure 6: Overview of in-vitro wounding assays (A) scratching, (B) stamping, (C) solid barrier, (D) liquid barrier, (E) droplet chemical assay, (F) microfluidic chemical assay, (G) electrical assay, and (H) laser ablation¹⁰⁶

Mechanical stamping systems employ rigid stamps to create multiple identical wounds simultaneously with less coating damage compared to scratching-based assays. In contrast, thermo-mechanical systems combine pressure with localized heating but create gradient zones where heat diffusion causes undefined gap sizes ¹⁰⁹. Electric current-based wounding applies localized electrical fields causing pH changes and membrane depolarization, but creates irregular wound areas and fails to fully detach strongly adhering cells like fibroblasts or keratinocytes ¹¹⁰. Finally, laser photo-ablation systems achieve precise cellular removal with micrometer-scale accuracy through controlled photothermal damage but are costly and therefore not easily accessible to smaller research groups ^{111,112}.

1.4 Co-Culture Assays for Vascular Mechanobiology

Vascular co-culture models enable investigation of complex cellular interactions within native vascular environments. Co-culture systems with appropriate measurement methods provide platforms to study these phenomena under controlled conditions, as endothelial cell dysfunction contributes significantly to cardiovascular disease progression through impaired cellular communication and paracrine signalling with smooth muscle cells ⁸⁴. Endothelial and smooth muscle cells communicate to regulate vascular development and function, making their co-culture essential for understanding vascular biomechanics.

In healthy blood vessels, endothelial cells form monolayer barriers regulating vascular function, while smooth muscle cells provide structural support and contractility ¹¹³. Modern vascular co-culture systems have evolved beyond simple two-dimensional monocultures to incorporate three-dimensional and layered configurations that better replicate blood vessel organization. Co-culture assays where endothelial cells are cultured in physical contact with smooth muscle cells, offer advantages for investigating contact-dependent cellular interactions and contractile behaviours allowing direct transmission of mechanical and biochemical signals between cell types ^{114–116}. Direct co-culture models align well with biomechanical studies investigating vascular contractility in response to vasoactive substances, enabling monitoring of contractile responses while preserving cell-cell interactions and mimicking in-vivo vessel geometry. For mechanobiological studies of cellular contractility under controlled mechanical environments, these co-culture systems on flexible substrates remain highly effective ¹¹⁶.

Culture substrate mechanical properties critically determine cellular behaviour within co-culture systems. Changes in microenvironment stiffness are involved in blood vessel pathological processes, with substrate stiffness having extensive effects on vascular endothelial and smooth muscle cells ^{7,116}. The CellDrum Technology™ provides an ideal platform for vascular co-cultures due to the tuneable mechanical properties and biocompatibility, while controlled mechanical stimulation systems enable investigation of how mechanical forces influence vascular cell interactions and tissue development.

1.4.1 Effects of Noradrenaline on Vascular Smooth Muscle Cells

Noradrenaline (NA) is a key sympathetic neurotransmitter regulating vascular tone, with vasoconstriction as its predominant physiological effect on VSMCs ¹¹⁷. This response is mediated primarily through adrenergic receptors, notably the α_1 -adrenoceptors (α_1 -ARs), which are highly expressed in arteries and arterioles. Upon activation, these G_q protein-coupled

receptors initiate signalling cascades that increase intracellular calcium levels, promoting actin-myosin interactions and resulting in smooth muscle cell contraction and a reduction in vessel diameter. Among these receptors, the α_{1A} and α_{1D} subtypes play a particularly significant role in mediating vasoconstriction^{113,117,118}.

α_2 -adrenoceptors (α_2 -ARs), while present in lower abundance, are also expressed in smaller vessels and contribute to vasoconstriction and smooth muscle cell proliferation. However, the role of α_2 -ARs in vascular regulation is complex, as systemic administration of α_2 -agonists like clonidine can paradoxically induce vasodilation in clinical contexts, reflecting the intricate regulatory role of adrenergic signalling in vascular homeostasis^{113,119}.

In contrast, β_2 -adrenoceptors (β_2 -ARs), found more prominent in skeletal muscle vasculature and coronary arteries, are coupled to G_s proteins and cause vasodilation through cAMP signalling pathways, leading to smooth muscle relaxation. Despite this, noradrenaline's higher affinity for α_1 -adrenoceptors ensures that vasoconstriction generally remains the dominant response, particularly in large arteries^{120,121}. However, the distribution of adrenergic receptors varies across vascular beds, allowing for dynamic regulation of vascular tone. Resistance arteries and arterioles exhibit a balance between α_1 - and β_2 -adrenergic activity, while specialized vascular regions such as the coronary and skeletal muscle circulations utilize β_2 -mediated vasodilation during increased physiological demand, even though coronary artery SMCs still express α_1 -adrenoceptors alongside lower levels of β_2 - and α_2 -receptors¹²².

Noradrenaline's concentration-dependent effects are particularly relevant, as lower concentrations can preferentially activate β_2 -adrenoceptors, leading to vasodilation, whereas higher concentrations lead to α_1 -adrenoceptor activation and vasoconstriction^{102,120,122}. This concentration-dependent receptor engagement allows noradrenaline to finely modulate vascular resistance according to physiological needs. In this thesis, the application of NA to SMCs cultured on ultra-thin PDMS membranes within the CellDrumTM system served to investigate contractile responses under defined mechanical conditions. Monitoring changes in membrane tension upon substance exposure, providing insight into how adrenergic signalling translates into functional contractile behaviour in vascular smooth muscle cells in a reproducible in vitro environment.

1.4.2 Effects of Noradrenaline on Vascular Co-cultures

Endothelial cells (ECs) and VSMCs interact closely in the vessel wall to regulate vascular tone, barrier function, and homeostasis. Direct co-culture systems, where ECs and SMCs are grown in contact on a shared substrate, provide a physiologically relevant model to investigate these interactions under controlled conditions^{114,116}.

Noradrenaline primarily induces vasoconstriction in SMCs through the activation of α_1 -adrenoceptors, with its effects being concentration dependent. At lower concentrations, noradrenaline can elicit mild contractions, whereas higher concentrations lead to stronger contractions, typically following a sigmoidal dose-response curve¹⁰². The degree of this response varies between vascular beds, reflecting differences in adrenergic receptor density and sensitivity across the vasculature. In coronary arteries, for example, vasoconstriction generally remains the predominant effect, although at lower noradrenaline concentrations, endothelium-

dependent vasodilation can become more pronounced, illustrating the interplay between smooth muscle contraction and endothelial modulation in regulating vascular tone .

Endothelial cells can modulate these responses through β_2 -adrenoceptor activation, which promotes nitric oxide (NO) synthesis and release. NO diffuses to the underlying SMCs, leading to smooth muscle relaxation through cyclic GMP signalling pathways. Additionally, α_2 -adrenoceptor activation in the presence of intact endothelium can contribute to vasodilation by stimulating the release of NO, prostacyclin, or endothelium-derived hyperpolarizing factors (EDHFs) ^{123,124}. In vitro studies, such as those investigating dexmedetomidine, have demonstrated a biphasic response in isolated arteries where an initial vasodilation at low concentrations, mediated by α_2 -adrenoceptors and dependent on nitric oxide and the endothelium, is followed by vasoconstriction at higher concentrations, indicating that this modulation is both time and concentration dependent ¹²⁴. This observation highlights the potential for other adrenergic agonists, including endogenous catecholamines like noradrenaline, to also exhibit such complex, context-dependent vascular effects.

Besides the previously described SMC monoculture experiments, direct co-cultures (EC-SMC) were also investigated on CellDrumTM membranes to examine the effects of noradrenaline in a more physiologically relevant model. This approach aimed to determine whether receptor-mediated responses, including the interplay between endothelial modulation and smooth muscle contraction, could be detected through changes in membrane tension. This experimental setup further allowed the evaluation of the CellDrumTM as a cell culture platform and its capacity to capture physiologically meaningful contractile behaviour in co-culture settings, supporting its potential use for investigating vascular pharmacological and mechanobiological responses.

1.5 Biomechanics of the Extracellular Matrix

While wound healing demonstrates the importance of mechanical forces and cellular responses, mechanical properties of the cellular microenvironment play equally important roles in determining cell behaviour and tissue formation. Substrate stiffness, defined as the rigidity of materials or surfaces on which cells reside, is a fundamental regulator of cellular functions for various biological processes ⁶. The ECM serves critical functions in this mechanical environment by ensuring force transmission within cell clusters and binding cells to surfaces, mediating the interface between cellular forces and mechanical properties of the substrate.

The primary mechanosensing apparatus involves focal adhesions that physically connect ECM to intracellular cytoskeleton ⁷⁸. When cells exert forces on substrates, the resistance encountered provides information about the environment's mechanical properties. The focal adhesion complex contains mechanosensitive proteins including FAK, paxillin, vinculin, and talin, which undergo conformational changes in response to mechanical forces, triggering downstream signalling cascades and regulating cytoskeletal dynamics ^{4,7}. Mechanosensitive ion channels also contribute by allowing Ca²⁺ influx that influences signalling pathways ^{79,81}.

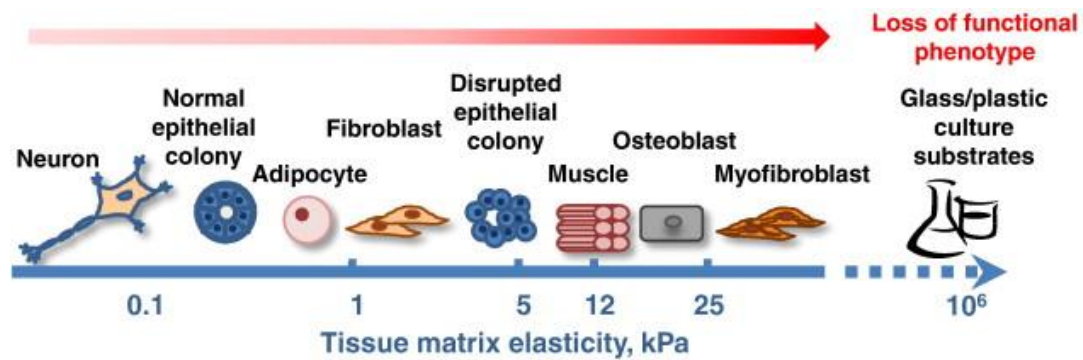


Figure 7: Overview of cell types and their corresponding matrix elasticity compared to classic culture flasks ³

On stiffer substrates, cells exhibit enhanced adhesion and pronounced stress fiber formation with elevated intracellular tension, while softer substrates promote more rounded morphologies with less organized cytoskeletal structures. For fibroblasts specifically, stiffer matrices promote larger, more stable focal adhesions, leading to increased cellular tension and proliferation ^{5,6}. Furthermore, cells on stiffer substrates migrate more slowly but with greater directional persistence, while softer substrates promote faster but less directed migration through a phenomenon called “durotaxis” (Lo et al., 2000) ¹²⁵. Conventional culture substrates often fail to replicate physiological tissue mechanical properties, leading to altered cell behaviour. Cells cultured on substrates with elasticity similar to native tissues maintain differentiated phenotypes and exhibit appropriate functional behaviours ^{3,4,6}.

One of the most remarkable aspects of substrate stiffness effects is its ability to direct stem cell differentiation and maintain cell phenotypes. Mesenchymal stem cells cultured on substrates with different stiffness values can be directed toward different lineages. While soft substrates (0.1-1 kPa) enhance neuronal differentiation, intermediate stiffness (8-17 kPa) favours myogenic differentiation, and stiff substrates (25-40 kPa) promote osteogenic differentiation ¹²⁶. For fibroblasts, substrate stiffness regulates their activation and differentiation into myofibroblasts, a process especially important for wound healing, requiring a threshold of 8-50 kPa depending on location. Below this range, fibroblasts remain less contractile and fail to upregulate α -smooth muscle actin (α -SMA). With sufficient mechanical resistance, cells adopt myofibroblast phenotypes capable of generating forces necessary for ECM remodelling and wound closure ^{66,127,128}. Optimal re-epithelialization occurs on intermediate stiffness substrates demonstrated by Huang et al. using axolotl skin explants (2-50 kPa), with softer substrates promoting pro-inflammatory responses while stiffer substrates support anti-inflammatory conditions and enhanced matrix production ¹²⁹.

Clinical relevance of substrate stiffness extends to pathological conditions where altered tissue mechanics impair healing. Chronic wounds often exhibit abnormal stiffness, preventing proper cellular responses. Yet, the relationship between matrix rigidity and healing is more complex than stiffness-dependent proliferation alone. While softer substrates may reduce proliferation in culture, they better replicate physiologically relevant biomechanics ⁸. Integrating mechanical compliance with dynamic stimulation may represent a better strategy to enhance healing. Culturing cells on ultra-thin membranes with controlled deformation could better mimic in-vivo wound environments. Such combination of compliance and cyclic stimulation as mentioned with the CellDrumTM Technology, might enhance mechanotransduction, potentially

improving the outcomes compared to rigid substrates, even when accounting for baseline proliferation differences.

1.6 Aims of Thesis

The primary aim of this thesis was to further develop, optimize, and validate the CellDrum™ technology as a versatile platform for cellular mechanobiology. A central focus was refining the technical aspects of membrane production and standardization to ensure reproducibility and mechanical consistency as the foundation for reliable experimentation. In parallel, the thesis aimed to test and demonstrate the biological applicability of the system by evaluating cellular responses to physiologically relevant mechanical conditions when cultured on the CellDrum™ membrane.

This approach was driven by the limitations of existing in vitro cell culture substrates, which often fail to replicate the complex and dynamic mechanical environments that cells encounter in-vivo. Conventional culture substrates are typically far stiffer than native tissues, and many stimulation systems are technically demanding with poor standardization. The CellDrum™ provides an alternative by combining ultra-thin flexible membranes with tuneable mechanical properties, precise stimulation capabilities, and real-time force measurement. Establishing such a platform is essential for advancing mechanobiology research, drug screening applications, and regenerative medicine, while offering a potential alternative to animal organ-based assays.

The work was organized into three interconnected phases, each addressing specific technical and biological challenges associated with the system.

Phase 1: Membrane Optimization, Characterization and Quality Control Development

The first phase involved optimization and characterization of the CellDrum™ membranes through deflection analysis and compliance measurements. This approach included fine-tuning the membrane production process to achieve reproducible mechanical properties and chemical functionalization protocols. The study examined whether significant correlations exist between membrane thickness variations and compliance characteristics within the narrow thickness range of 3.2-4 µm.

The investigation included analysis of manufacturing parameters and process validation to determine whether thickness measurement is necessary as a quality management parameter. This evaluation led to the development of standardized quality control procedures that evolved into standard operating protocols (SOPs), ensuring reproducibility across different experimental conditions. This approach further ensured consistent production of membranes with defined thickness parameters and mechanical properties. The membranes were chemically prepared for subsequent biological functionalization steps, including protein coating applications to enhance cell adhesion and optimize culture conditions.

Phase 2: Biological Application Validation

The second phase focused on validation of the optimized CellDrum™ system through biologically relevant wound healing assays using primary dermal fibroblasts. This validation included development of a standardized wound healing assay using fibroblasts from donors of different ages (28 and 88 years old), allowing investigation of age-related differences in cellular

responses and wound healing kinetics. As mentioned before, fibroblasts are key players in tissue repair, and their age-dependent differences make them an important model for studying impaired wound healing.

A technical challenge was developing a novel wounding method adapted for the ultra-thin CellDrum™ membranes. Due to the membrane's delicate nature, conventional wounding techniques such as scratch assays or thermal/electrical applications were unsuitable as they could damage or destroy the membrane structure. A novel wounding technique was developed specifically for the CellDrum™ membranes, using stainless-steel spheres to create reproducible circular cell-free gaps without damaging the substrate. This approach preserved membrane integrity while achieving reproducible wound geometries.

The wound closure process was monitored over time and compared between fibroblasts from young and aged donors. Integration of biaxial cyclic stretching through the PulSelect system enabled investigation of mechanically stimulated versus non-stimulated cellular responses during wound closure, providing insights into mechanotransduction mechanisms during tissue repair. This experimental design tested whether controlled mechanical training could accelerate closure in young fibroblasts and reveal distinct, potentially impaired responses in aged cells, thereby highlighting the platform's sensitivity for detecting biologically and clinically relevant mechanobiological differences.

Phase 3: Advanced Cell Monolayer and Co-culture Applications

The third phase focused on establishing the CellDrum™ system's capabilities for three-dimensional co-culture applications and vascular tissue modelling. This involved developing protocols for cultivation of smooth muscle cells (SMCs) and endothelial cells (ECs) from coronary and aortic origins in layered configurations that model native tissue architecture. The co-culture approach was designed to mimic the cellular interactions and mechanical environment found in blood vessel walls, where endothelial cells form the inner lining and smooth muscle cells comprise the medial layer. Special attention was given to maintaining the distinct phenotypic characteristics of each cell type while ensuring proper cell-cell communication and tissue-like organization.

Following co-culture establishment, validation was performed through immunofluorescent staining and microscopic imaging to confirm cellular organization, viability, and phenotypic characteristics. Specific markers were used to distinguish between cell types and assess their differentiation state and functional properties. The validated system was used to investigate cellular responses to vasoactive substances like ionomycin and noradrenaline, with real-time measurements of membrane tension changes as indicators of cellular contractile responses.

This phase demonstrated the platform's capability to support complex tissue models and measure physiologically relevant responses to pharmacological agents. The ability to monitor contractile forces in real-time provided insights into vascular reactivity and the mechanisms underlying vasoconstriction and vasodilation, establishing the system's potential for drug screening and cardiovascular research applications.

In summary, the overall goal of this thesis was to establish the CellDrum™ system as a platform for biomechanical testing applications. Through systematic optimization and validation protocols, this research demonstrated the system's applicability across experimental paradigms including wound healing studies, mechanotransduction research, and vascular biology investigations. This platform shows tremendous potential to replace traditional animal organ-based research methods, including vascular ring assays, myocardial strip preparations, and ex-vivo skin wound models. By providing a more controlled and reproducible alternative, this technology advances the field toward more standardized experimental approaches while reducing reliance on animal-derived tissues. The development and validation of the CellDrum™ system represents an advancement in cellular mechanobiology research, offering researchers new capabilities for investigating mechanical aspects of cellular behaviour in a controlled and reproducible manner.

2. Material and Methods

2.1 Material and Instrument List

Table 1: List of materials used

Material	Brand	Product No.
(-)-Norepinephrine (Noradrenaline)	Sigma Aldrich	A7257
0.05% Trypsin/EDTA	Gibco	
2-Propanol (Isopropanol), 99.8%	Carl Roth	6752.1
Acetic Acid, 98%	Carl Roth	
Alexa Fluor™ 488 Phalloidin	Invitrogen	A12379
Anti-human CD31, AF594, Mouse, Monoclonal	Biolegend	BLD-303126
α-smooth Muscle Actin, AF488 Monoclonal Antibody	Thermo Fischer	53-9760-82
Bovine Fibronectin	Sigma Aldrich	F1141
Bovine Serum Albumin		
CellTracker® Green CMFDA dye	Invitrogen	
DMEM + Glutamax	Gibco	
Dimethyl sulfoxide (DMSO)	Sigma Aldrich	D8418
Dulbecco's Phosphate Buffered Saline (DPBS)	VWR	
Endothelial Cell Growth Kit-VEGF	ATCC	PCS-100-041
Fetal Bovine Serum (FBS)	Gibco	
Hoechst nucleic acid stain	Thermo Fischer	33342
Human SMC Differentiation Medium	Cell Applications	311D-250
Hydrochloric acid, 37%	Carl Roth	4625.1
Hydrogen peroxide solution, 30%	Sigma Aldrich	H1009
Ionomycin Calcium Salt	Sigma Aldrich	I3909
Live-Dead Staining		
Paraformaldehyde		
Penicillin-Streptomycin-Amphotericin B Solution	ATCC	PCS-999-002
Trimethoxy[2-(7-oxabicyclo[4.1.0]hept-3-yl)ethyl]silane	Sigma Aldrich	413321
Triton-X	Sigma Aldrich	45H0481
Trypan Blue	Sigma Aldrich	
Trypsin Neutralizing Solution	Pellobiotech	
Vascular Cell Basal Medium	ATCC	PCS-100-030
Vascular Smooth Muscle Cell Growth Kit	ATCC	PCS-100-042

Table 2: List of cell lines used

Cell Lines	Lot Number	Company	Extracted Region
HAOEC	PCS-100-011	ATCC	Aorta
HAOSMC	PCS-100-012	ATCC	Aorta
nHDF adult 28 years-old	490824	LONZA	Abdomen
nHDF adult 88 years-old	664504	LONZA	Abdomen

Table 3: List of used devices

Instruments Used	Brand
Automated Cell Counter TC-20™	Bio-Rad
Centrifuge-Biofuge Primo	Heraeus
CFA _{refer} , Cell Force Analyzer	-
F20 Film Thickness Measurement System	Filmetrics
Fluorescence Microscope, BZ-8100E	Keyence
Laser Triangulation System	-
Phase Contrast Microscope, Axiovert 25	Zeiss
PulSelect, cell trainer device	-

2.2 Innovative Instrumentation

This section presents the instrumentation developed within the laboratory at the University of Applied Sciences Aachen, Center of Competence in Bioengineering, that formed the foundation of the experimental work in this thesis. These devices were specifically engineered to address the unique requirements of mechanobiology research, enabling precise control and measurement of cellular responses under defined mechanical conditions.

2.2.1 Laser Triangulation System

For direct measurements of the membrane deflection, a prototype Laser Triangulation System (Figure 8) equipped with a laser triangulation sensor was used¹⁸. A customized graphical user interface programmed with LabVIEW® (LabVIEW 17.0.1f3, TX, USA) was available in the laboratory for data acquisition.

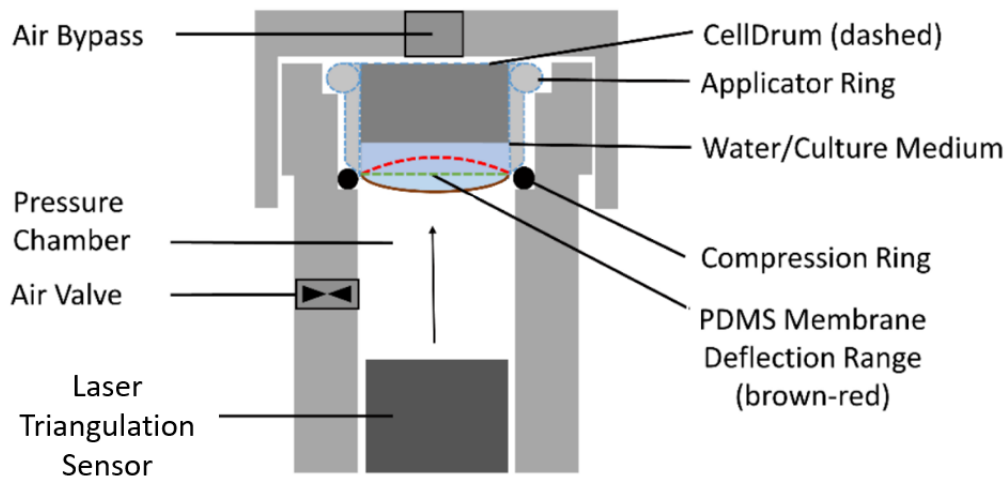


Figure 8: Schematic cross-section of the Laser Triangulation- deflection measurement system. The contour of the CellDrum™ is shown dashed in blue. The PDMS membrane forms the lower part of the CellDrum™. The figure indicates three membrane positions: Brown and red mark the lower and upper limits, respectively, of the membrane bending range used for the measurement. Green marks the flat membrane condition, i.e., the strain minimum. The membrane curvature is determined by the pressure in the pressure chamber, the membrane properties (PDMS membrane with attached cell layer) and the weight of the water/medium above the membrane.

To directly measure membrane deflection by light reflection, a single CellDrum was inserted into the ring socket and 500 μl PBS was pipetted onto the membrane surface as a hydrostatic load. This caused the membrane to bend downward which was measured with the laser triangulation sensor. Prior to measurements, fine talcum powder was applied to membrane centers using a soft stamp to create non-transparent spots for laser signal detection. A blank measurement was taken for calibration before each deflection measurement.

2.2.2 CFA_{refer}

The deflection measurement described in Section 2.2.1 was utilized only for preliminary characterization of membrane mechanical properties until the CFA_{refer} device became available. While this method provided valuable baseline data on membrane elasticity, it presented significant limitations unsuitable for routine quality control and cell culture applications. The requirement for talcum powder application prevented membrane reuse for cell culture due to contamination concerns and interfered with phase contrast microscopy visualization. Additionally, manual PDMS membrane production variability necessitated individual testing that was impractical with the talcum powder method. The CFA_{refer} records pressure changes that occur when defined volumes of air are introduced into the pressure chamber, rather than measuring indentation directly. From these data the mechanical membrane tension can be calculated and presented in mN/mm^2 . This approach also requires no surface modification or contaminating substances, enabling non-destructive testing suitable for cell culture applications

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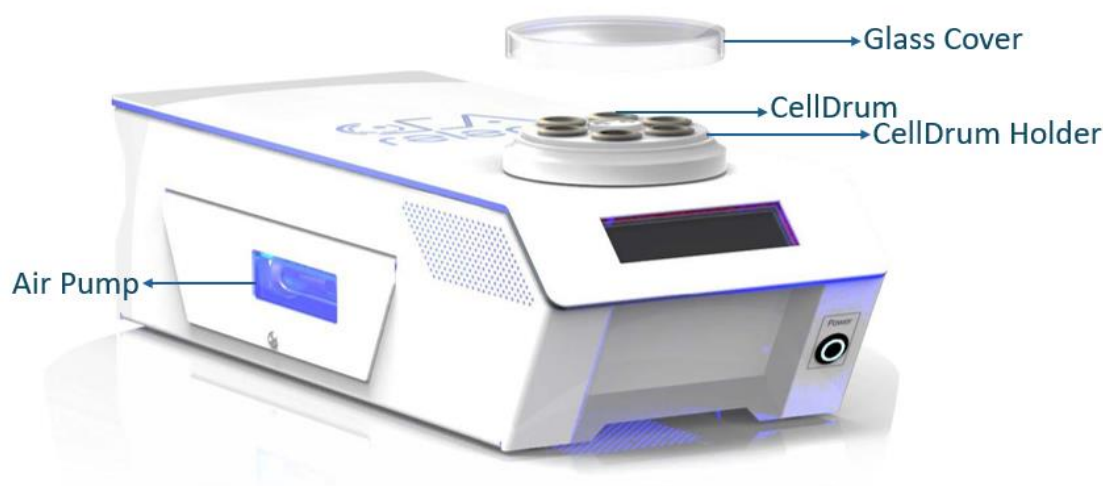


Figure 9: CFA_{refer} features a stainless steel housing that can be covered with a Petri dish cover (Glass/Plastic). The removable CellDrumTM holder can be autoclaved for sterile workflows and accommodates up to six CellDrums simultaneously. A built-in pump delivers precise air pressure, while an integrated heating element with PID control maintains physiological conditions (37 ± 0.2 °C).

The CFA_{refer} instrument (Figure 9) is a refined, user-friendly, and compact tabletop system designed for the precise measurement of mechanical responses of cell layers cultured on CellDrumTM membranes. Its compact size allows easy integration into smaller laboratories, and its design enables straightforward cleaning and maintenance. The instrument is connected to a laptop, which controls the measurement process via a desktop application (Figure 10) and enables data processing in specialised software.

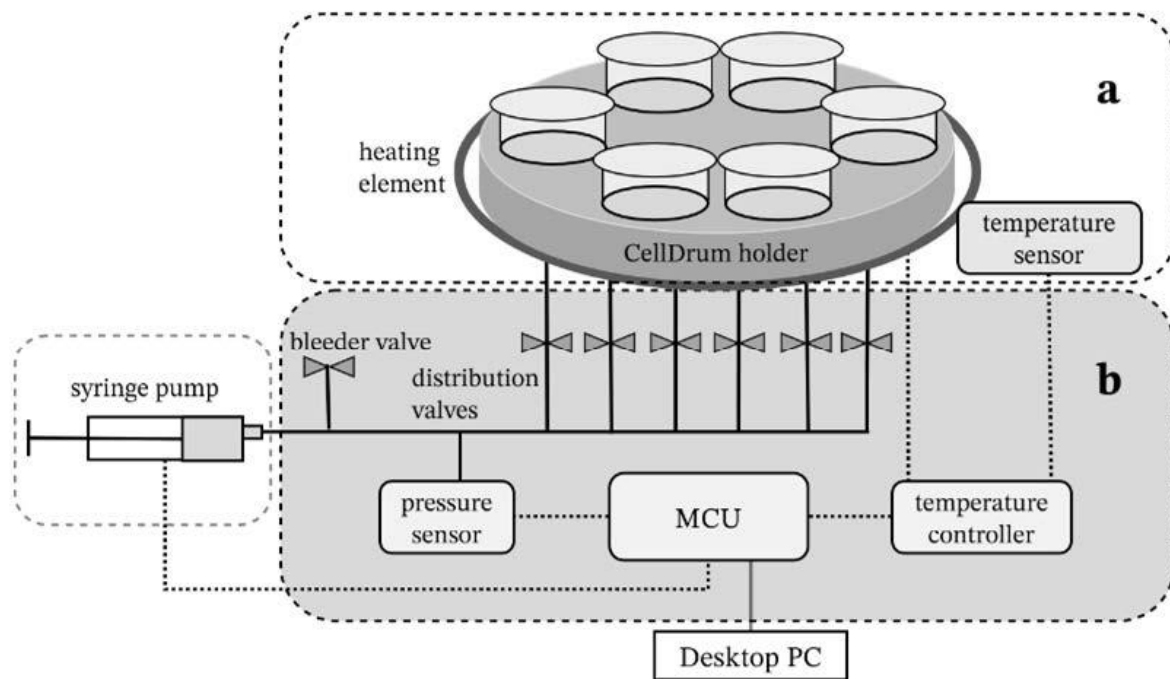


Figure 10: CFA_{refer} measurement scheme, a) CellDrumTM holder and CellDrums b) internal components

Figure 10 shows a diagram of the internal structure of a CFA_{refer} . An array of valves mounted to a manifold distributes the pumped air to each of the six wells individually, with an additional bleeder valve included to release overpressure during CellDrumTM holder insertion.

Prior to starting a measurement with the CFA_{refer} , the user is required to input specific parameters into the system to enable accurate data acquisition (Figure 11). The volume of the culture medium in each well must be entered, and the membrane thickness of the CellDrumTM must be specified to ensure precise calculation of membrane tension. At least one measurement channel must be activated for the measurement sequence to begin. While other parameters, such as sample ID, medium type, and supplement type or volume, are optional, it is recommended that these details are provided for organized data management in post-processing.

Position	Prefix	CellDrum ID number	CellType	Medium	Volume [μL]	Membrane Thickness [μm]	Supplement Name	Supplement Volume [μL]	Comment
(1)									
(2)									
(3)									
(4)									
(5)									
(6)									

Figure 11: CFA_{refer} user interface for measurement setup tab

The air pressure under the membrane of each individual CellDrum is increased separately and continuously during the measurement. At the same time, the pressure values are recorded so that the system can quantify the force response of the cell membrane system and output mechanical stress data in mN/mm^2 . The user can adjust the applied pressure value up to a maximum of 1 ml, limited by the volume of the integrated syringe pump, to meet specific experimental requirements.

At this point, the following should be noted regarding the membrane tension in mN/mm^2 :

1. The value indicates the total mechanical tension of the membrane, the cultured cell layer, including the extracellular matrix. From a technical standpoint, this is referred to as a composite membrane.
2. All cells adhering to the membrane contribute to the total mechanical tension of the composite membrane. The contraction force of the individual cells multiplied by the total number of cells on the membrane is the contribution of all adhered cells to the total mechanical tension. If this is to be determined, it would be essential to remove the cells from the membrane after measurement and count them separately. This way the measured mechanical stress in mN/mm^2 can be divided by the number of cells involved. We only then obtain the average mechanical stress exerted by a single cell. When comparing cell forces measured using different methods with data in the literature, this must be taken into account. This is simply not an option with other measurement methods and is therefore disregarded as it leads to physically questionable data on the mechanical stress of contractile cell layers or "cell structures". Relative values are then often given, and these must be questioned for other reasons^{131,132}.

These technical explanations coarsely summarize how the CFA_{refer} works. Further technical, physical and biological/pharmacological details are currently being worked out in another PhD thesis¹³³.

2.2.3 PulSelect

Cellular responses to external mechanical forces are highly sensitive to parameters such as amplitude, frequency, duration, and the mechanical properties of the experimental substrate, where even small variations can result in significantly different biological outcomes. To address this, the development and implementation of standardized protocols are essential, especially when using advanced experimental platforms designed to simulate physiological mechanical environments.

Due to the limitations of uniaxial systems, a device that accommodates the CellDrumTM system and enables customizable biaxial cyclic mechanical stimulation across a range of frequencies and amplitudes was developed at the University of Applied Sciences Aachen, Center of Competence in Bioengineering (Figure 12). In this mechanical stimulation system, named PulSelect from this point on, CellDrums serve as a flexible growth surface that allow cyclic external simulation with controlled biaxial mechanical loads. This setup allows us to mimic a wide range of in vivo-like mechanical environments while offering a robust framework for investigating mechanotransduction processes^{23,24}. The following section outlines the structure, functionality, and operational principles of this experimental setup.

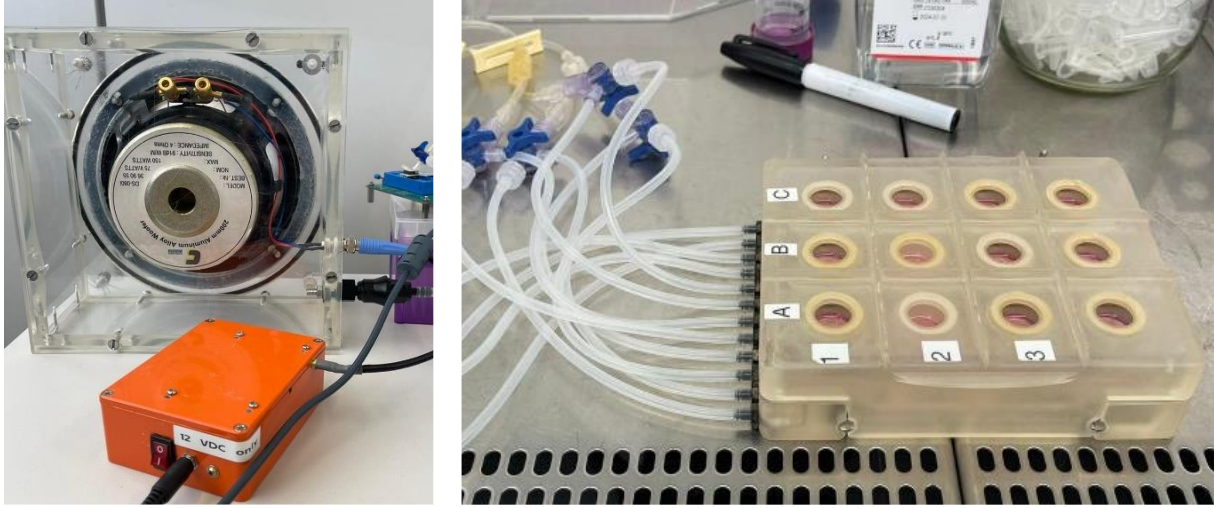


Figure 12: Experimental setup showing the frequency generator and loudspeaker assembly (left) coupled with the PulSelect CellDrum™ holder system (right).

The PulSelect introduces biaxial stress to CellDrums through frequency-induced oscillations. The setup consists of five main components: a loudspeaker that converts electrical signals into mechanical oscillations, a frequency generator to produce oscillatory signals, a frequency distributor for signal management, a signal visualization display for real-time monitoring, and the CellDrum™ holder where mechanical stimulation occurs. Some components are positioned within a sterile workbench to avoid contamination, while others remain external.

As a result of other experiments performed in our laboratory at FH Aachen, a cyclic stimulation of 1 Hz was shown to create a detectable ECM remodelling without harming the cell monolayer. Therefore as training parameters for the experiments in this thesis, a frequency of 1 Hz with a peak-to-peak voltage (V_{pp}) of 10 was selected. The strain amplitude was indirectly adjusted by setting the peak-to-peak voltage of the frequency generator to 10V. This resulted in an average pressure amplitude of 7Pa. With the help of a membrane deflection model, an approximate strain value of 5% was calculated.

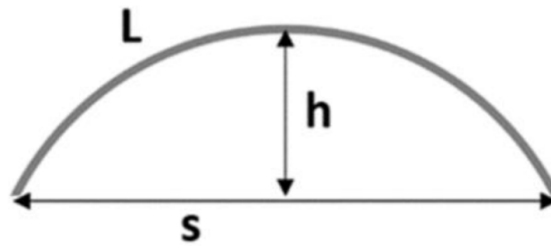


Figure 13: Graphical representation of the membrane deflection model. Value s , or the span of the circular arch, is the inner CellDrum™ applicator diameter. Value h is the deflection that can be measured by distance sensors.

Taking into consideration that the membrane is axisymmetric, the membrane strain can be calculated in a two-dimensional model. The general formula for calculating the strain is given below (Equation (1)).

$$\varepsilon \text{ (strain)} = \frac{\Delta L}{L_0} \quad (1)$$

While L_0 is the default length, ΔL stands for the change of length. In the case of the CellDrum™ membrane, the length of the cross-sectional arch is taken as L . This arch is approximated to a circular arch with the formula (Equation (2)).

$$L = \frac{\arctan\left(\frac{2h}{s}\right) * (4h^2 + s^2) * \frac{\pi}{180}}{2h} \quad (2)$$

The average downward deflection h of a CellDrum™ membrane when solely loaded with cell culture medium is obtained empirically. With Equation (2), one can calculate the corresponding length of L_0 . Since the very low-pressure readings did not allow for a more precise calculation of the cellular strain, the focus was laid on pulse frequency and duration.

2.3 CellDrum™ Production and Quality Control

2.3.1 CellDrum™ Applicator Material Selection

Several alternative materials were evaluated to optimize the CellDrum™ applicator with respect to mechanical stability, ease of use, surface roughness, and cost-effectiveness in production. Polymethylmethacrylate (PMMA) had been tested in earlier work within the laboratory; however, it did not fully meet the mechanical and operational requirements of the system. During the course of this thesis, Polypropylene Homopolymer (PP-HOMO) was investigated as a less expensive yet durable alternative to PEEK, particularly with a view toward potential future commercialization of the system.

PP-HOMO underwent biocompatibility assessments, and surface characterization using scanning electron microscopy (SEM), which was performed by the Gemeinschaftslabor für Elektronenmikroskopie (GFE) in Aachen. A sample from each material was labelled according to their positions in a holder, with the PEEK material designated as sample 1 and the PP-HOMO as sample 6. Two detection systems were employed during SEM analysis: Variable Pressure Secondary Electron (VPSE) and Backscattered Electron Detector (BSD4 A).

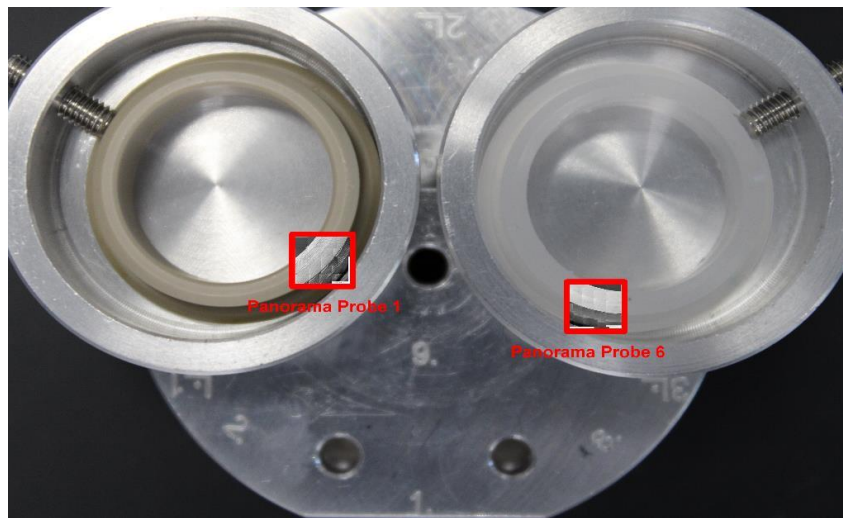


Figure 14: Labeling of the samples: PEEK - Sample 1 and PP - Sample 6

VPSE enabled the acquisition of topographical images with enhanced low kV imaging and increased scan speed, while the variable pressure conditions (~10 Pa to 3,000 Pa) allowed for the imaging of samples without conductive coatings, which is advantageous when coating is undesirable or impractical. The BSD detected elastically scattered electrons, providing imaging with sensitivity to differences in atomic number, where heavier elements appear brighter, and reducing the need for extensive sample preparation while minimizing potential beam damage.

Later on, to assess the biocompatibility of PP-HOMO, four 6-well plates were divided into four groups, with two plates serving as control groups and two as experimental groups. Each plate was used to test both dosages (2 material pieces [2M] and 4 material pieces [4M]) over different time frames, with 2-day and 4-day incubation periods, respectively. The control groups, although labelled with material numbers for grouping consistency, did not contain any material to serve as negative controls.

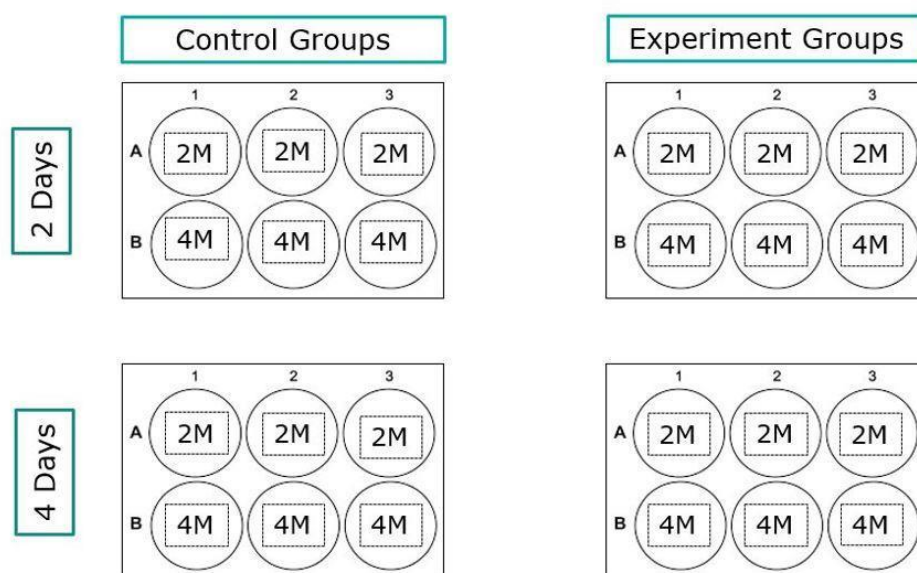


Figure 15: Experimental setup for biocompatibility testing

The experimental procedure was performed as follows:

1. nHDFa cells were cultured and counted, with 1×10^5 cells seeded into each of the 24 wells. Twenty-four hours post-seeding, the first medium change was performed, followed by medium changes every 48 hours using DMEM supplemented with 10% FBS. After a 2-day proliferation period to ensure cell viability, sterilized PP-HOMO material pieces were added to the wells of the experimental groups according to the dosage scheme.
2. Following 2 and 4 days of material exposure, the cells were detached using 0.05% Trypsin-EDTA, and cell counts and viability were assessed using Trypan blue staining and a counting chamber. The results were analysed statistically, comparing cell counts and viability rates between the experimental and control groups to determine the material's biocompatibility.

At the end of material testing, although some prototype holders were successfully produced with PP-HOMO and the material exhibited biocompatibility and heat resistance, it was not used for the experiments described in this thesis due to the continued availability of a sufficient stock of PEEK holders. Furthermore, the CellDrum™-matching devices PulSElect and CFA_{refer} were designed with the PEEK-based CellDrums in mind.

2.3.2 CellDrum™ Membrane Production

A key objective of this thesis was optimizing and standardizing the CellDrum™ production process, which is prone to various errors due to its manual nature. Efforts focused on refining the procedure to minimize user-dependent variability and simplifying the workflow to enhance efficiency.

Since these membranes are intended for cell applications involving biomechanical measurements, ensuring consistent membrane quality is critical for reproducibility and comparability in experimental results. The standard operating procedure (SOP) for ultra-thin membrane production is as follows:

1. To prevent bubble formation on the water surface during the heat-curing, the water is subjected to vacuum treatment until no visible bubbles remain in the liquid.
2. The base and curing agent are combined in a 10:1 (w/w) ratio and centrifuged for 2 minutes at 3200 rpm. Due to differing viscosities, this initial centrifugation moves material adhering to tube walls towards the bottom. To achieve thorough mixing, the mixture is stirred once more using a sterile stirrer and centrifuged again to remove remaining air from the solution.
3. Once both the water and silicone mixture are prepared, 6-well culture flasks are placed under heating lamps, and each well is filled with 10 ml of bubble-free deionized water. Dependent on the required membrane thickness, a matching amount of silicone elastomer is pipetted onto the water surface using a positive displacement pipette, as centrally as possible to increase thickness homogeneity. To produce membranes with thicknesses between 3.2-4.0 μm , 4.15 μl of silicone elastomer was found suitable.
4. After all wells are coated with the PDMS elastomer mixture, the infrared heating lamps are turned on, and the silicone is cured for 30 minutes.



Figure 16: Membrane curing setup (R) with the custom metal stamping device with the transfer tool (L)

5. At the end of 30 minutes, the heating lamps are turned off, and the membranes are left to cool for around 15 minutes, before transferring them onto the CellDrumTM applicators. For the transfer, a custom made, 3D printed tool is used.
6. Prior to thickness measurement, the CellDrums are autoclaved to prevent uncured silicone remains, as the autoclave conditions (120 °C) provide an additional safeguard to the curing procedure. Membranes that are outside the predefined thickness range as well as membranes with visible holes will be excluded, and the selected membranes will then undergo further quality control procedures, as described below.

2.3.3 Membrane Functionalization

To create a hydrophilic surface for the biological coating, firstly a two-step wet-chemical functionalization protocol composed of an oxidation step and a silanization step was used^{134,135}. All used solutions were prepared freshly prior to membrane functionalization. The oxidation solution was prepared following the procedure by Sui et al. as H₂O/H₂O₂ (30%)/HCl (37%) were mixed in a 5:1:1 volume ratio¹³⁴. Membranes were exposed to the solution for 30 min, washed and the same step was repeated two more times, without waiting time between washes.

Next silanization step was performed with a 2% solution of trimethoxy [2-(7-oxabicyclo[4.1.0]hept-3-yl)ethyl]silane in an Isopropanol/Water (19:1) mixture with a pH of 4.9–5.0, regulated using acetic acid and was kept on the membrane surface for 5 min¹³⁵. The silanization solution is discarded, membrane is washed using 99% isopropanol to remove any remaining silanes before being washed again with distilled water and is then autoclaved at 121 °C, 0.2 MPa for 20 min to stabilize the previous silanization step.

For the biological functionalization, fibronectin from bovine blood plasma (Sigma Aldrich Chemie, Taufkirchen, Germany) was chosen as matrix coating. The ready-to-use solution was diluted down to 1% (v/v) concentration using a 50 mM, 2-(N-morpholino)ethanesulfonic acid (MES) buffer made with autoclaved deionized water¹³⁶. The buffer pH was set to 6.1 using 1 M NaOH solution and sterilized using a 0.22 µm filter. Finally, the fibronectin solution is removed after 24 h incubation time and the membranes are kept refrigerated with phosphate buffer saline solution (PBS) until use.

2.4 Membrane Quality Assessment

Quality management in membrane production requires systematic control of manufacturing processes and various testing protocols to ensure consistent product performance. Regular equipment calibration, documentation of process parameters such as room temperature and curing agent: base ratio and following an established standard operating procedures are essential components of the quality management system. This comprehensive approach guarantees that membranes meet specified performance criteria and maintain reproducible characteristics necessary for reliable experimental results.

2.4.1 Preliminary Visual Examination

All membranes used in experiments must undergo a preliminary visual examination before proceeding to further testing phases. This quality control step makes sure that only membranes with proper structural integrity and surface uniformity are used in experimental procedures.

During inspection, membranes must not have any wrinkles or surface deformation, especially at attachment sites. These defects can affect mechanical properties and skew stress measurements. The entire membrane surface must be examined for holes, punctures, or dust particles, which could create stress concentration points. Rejecting membranes with these flaws ensures uniform stress distribution during mechanical testing and proper sealing to prevent fluid leaks. Membranes that exhibit surface defects, structural irregularities, or contamination are rejected and excluded from further experiments. This pre-screening process serves two important functions: it eliminates defective specimens that would produce unreliable results, and it improves laboratory efficiency by reducing unnecessary work and shortening the overall duration of quality control testing procedures.

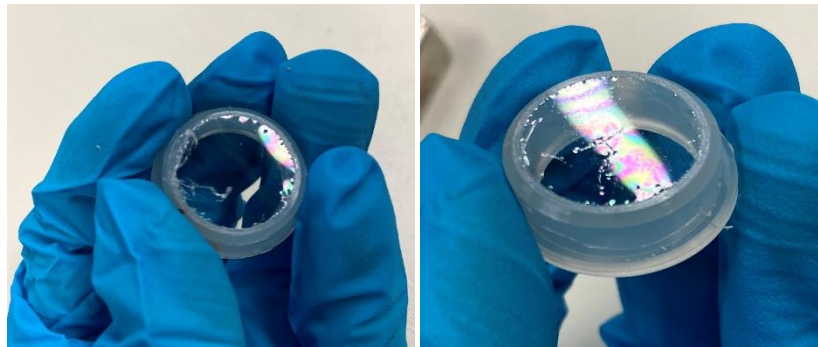


Figure 17: Possible defects on CellDrum™ membrane post-production

2.4.2 Membrane Thickness Measurement

To measure the membrane thickness of the thin PDMS films, Filmetrics F20 (KLA Instruments, San Diego, CA, USA) spectrometer was used.

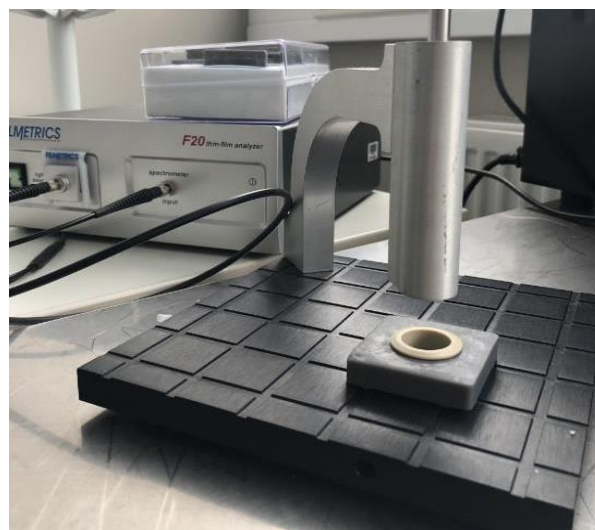


Figure 18: FILMETRICS F20 Thickness Measurement Device

Once the measurement is completed, the data are analysed by the comparison to a calculated reflectance spectrum, which is a function of the given material's refractive index. The setup also allows thickness determination of multiple layers through a “recipe” system, where the user must define the structural order of different materials. In the measurement of this thin PDMS membrane, the used recipe was “Air-PDMS-Air” as the thin film is free-standing, therefore not connected to any other material and is simply surrounded by air. The calibration of the device can be controlled with a thickness standard, also provided by the company Filmetrics, and the goodness of fit (GOF) can be regulated by adjusting the height of the lens which supplies the system with the light through fiber optic cables.

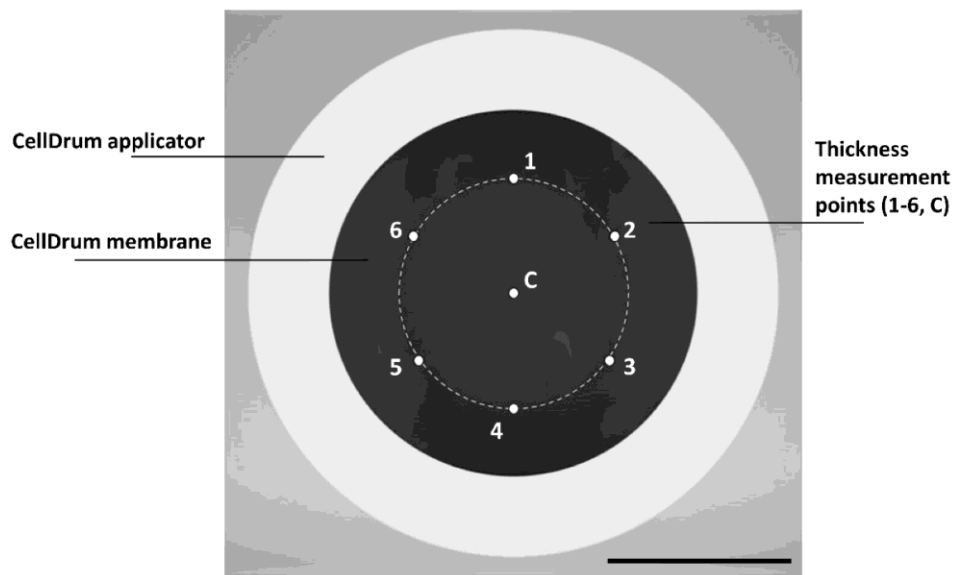


Figure 19: Positioning of thickness measuring points for CellDrumTM membranes (top view). Inner membrane diameter is 1.6 cm, and angular spacing of the data points on the measuring circle is 60°. Measurement points are numbered from 1–6 for eccentric measurements and C for central, respectively. The scale bar represents 8 mm in length.

Special holders were constructed and 3D-printed to ensure that the thickness measurement was performed at the same seven spots for each membrane. Eccentric aid allows the CellDrumTM to be rotated via a revolver which provides a 60° spacing between each measurement point to ensure thickness homogeneity of a batch if required. If no unusual reflection patterns are visible on the membrane, only centric measurement will be carried out.

Membrane thickness depends on the control of multiple parameters such as elastomer volume, mixing ratio, curing temperature and initial PDMS and water temperature before curing^{57,137}. For the investigation of thickness dependency on temperature, three different room temperatures were applied for testing (22,24 and 26 °C), using PDMS and water that were also stored in the same room before production, to keep the parameters as uniform as possible. Another parameter used to investigate the dependency of the final membrane thickness was the PDMS volume. For this purpose, three different PDMS volumes were tested and compared between each other.

2.4.3 Membrane Compliance Measurement

Mechanical compliance is a measure of flexibility of a material. The more compliant a material is, the more it gives in under load. Usually, the compliance of the CellDrum™ membrane is measured with the CFA_{refer} system. For that, the instrument slowly increases the pressure under the membrane deflecting it upwards. The pressurizing affects the strain within the membrane which can be indirectly determined by measuring the pressure under the membrane and the membrane's deflection. The compliance parameter was introduced in particular for the intuitive understanding of the measured data in biomedical studies¹³⁸.

$$\varepsilon \text{ (strain)} = \frac{\Delta_{Length}}{Length} \quad (1)$$

When plotting a graph of deflection over pressure, the measured data represent a sigmoid curve crossing the line of minimum strain. At the zero crossing point the compliance value is then acquired by conversion of the pressure and deflection value to receive the ratio of the change of strain to the change of stress, as depicted in the following formula:

$$C \text{ (compliance)} = \frac{\Delta_{strain}}{\Delta_{pressure}} \quad (2)$$

The measurement of CellDrum™ compliance or mechanical stress, as defined by Equations (1) and (2), can also be applied to evaluate the mechanical properties of a cell-membrane composite, in this case a PDMS membrane with an attached cell layer^{14,138,139}. Because the cells adhere tightly to the membrane surface, they contribute to its overall mechanical behaviour. This contribution is only detectable if the membrane is sufficiently thin, as previously described. Two key conditions must also be met: (1) membrane deflection must be measured with high precision under well-defined boundary conditions, and (2) the mechanical properties of each membrane must be accurately recorded before cell seeding. These baseline values are essential for correctly interpreting the mechanical response of the membrane with cells.

2.5 Cell Culture

2.5.1 Culture of Dermal Fibroblasts

Normal human dermal fibroblast (nHDFa) cell lines were purchased from LONZA, Germany, derived from abdominal tissue of 28 and 88-year-old female Caucasian donors. Below are the detailed steps followed for the used cell culture procedure.

1. Cells stored in liquid nitrogen at -189°C were rapidly thawed in a 37°C water bath according to manufacturer's instructions. Cell viability was assessed, and cells were counted using an automated cell counter. 3500 cells/cm² were seeded into T75 flasks containing 15 ml pre-warmed growth medium (10% FBS in DMEM, Glutamax). Medium was replaced the day after seeding and every two days thereafter.

2. Cells were passaged once before experimental use to regain healthy morphology. At confluency (passage 4), cells were detached using 0.05% Trypsin-EDTA for 5 minutes, then inhibited with growth medium.
3. After counting, 100,000 cells in 300 μ L DMEM with 10% FBS were seeded onto fibronectin-coated CellDrumTM membranes (2.01 cm² culture area). These were incubated under standard conditions (37°C, 5% CO₂) for minimum 24 hours for attachment and spreading. Cell attachment and confluency were assessed using inverted phase-contrast microscopy before mechanical training and analysis. These CellDrums with the fibroblasts (both age groups) were further used in cell stimulation experiments using the PulSelect device as explained in Section 2.5.

2.5.2 Culture of Smooth Muscle Cells and Endothelial Cells

Primary human aortic smooth muscle cells (HAOSMC) and human aortic endothelial cells (HAOEC) used in this study were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Both cell types are taken from the human aorta, ensuring physiological relevance for vascular mechanobiology experiments performed in this thesis.

1. Upon arrival, HAOSMC and HAOEC were thawed, seeded at 3000-5000 cells/cm², and maintained under optimal conditions (37°C, 5% CO₂) until reaching required confluency. HAOSMCs were used at passage 5, while HAOECs were used at passage 4.
2. Both cell types were maintained in standard ATCC culture medium. Medium was replaced 24 hours after thawing to remove cryoprotectants and dead cells, then changed every 48 hours until confluency.
3. At ~80% confluency, HAOSMCs were seeded onto CellDrums at ~50,000 cells/cm² and maintained in growth medium with 1% fibronectin for 2 days to allow attachment and enhance mechanical durability. After 2 days, medium was replaced with low-serum differentiation medium, and HAOSMCs were differentiated for 8-10 days with daily medium changes.
4. For monolayer experiments, differentiated HAOSMCs were used directly for noradrenaline stimulation and measurements. For co-culture experiments, HAOECs at ~80% confluency were seeded onto differentiated HAOSMC monolayers at ~75,000 cells/cm². Medium was switched to endothelial cell growth medium, and co-cultures were allowed to establish for 48 hours.
5. Throughout the culture period (Days 1-12), regular medium changes and morphology checks were performed using phase contrast microscopy. On Day 12, HAOSMCs were stained with α -smooth muscle actin (α -SMA, Alexa Fluor 488) as a differentiation control on one sample.

Differentiation of HAOSMC cells in culture flasks before seeding on CellDrumTM membranes was tested, however, cell counts were consistently lower than expected, resulting in suboptimal monolayers after seeding. Therefore, differentiation was performed directly on the membrane. While the company manual recommends 10-day differentiation, the CellDrumTM membrane's softer substrate compared to conventional culture flasks may allow for shorter differentiation periods.

Based on previous tests with other smooth muscle cell types using the same differentiation medium, fluorescence staining for α -smooth muscle actin (α -SMA) on day 8 confirmed successful differentiation. Therefore, the differentiation period was set to 8 days to minimize contamination risk from extended culture while ensuring effective differentiation on the CellDrum™ system.

2.5.3 Fluorescence Staining of Vascular Cells

Following co-culture preparation, fluorescence staining procedures were performed to confirm cell identity and phenotype prior to mechanical measurements. These procedures ensured HAOSMCs had gained a contractile phenotype while HAOECs retained endothelial-specific characteristics, excluding cross-contamination during cell collection.

For validation, two samples were prepared for each condition: HAOSMC monolayers, HAOEC monolayers, and HAOSMC-HAOEC co-cultures. Antibody specificity was evaluated by cross-staining to confirm HAOSMCs did not express CD31 and HAOECs did not show α -SMA. CD31 was detected using Alexa Fluor 647-conjugated antibody, while α -SMA was visualized with Alexa Fluor 488-conjugated antibody, enabling simultaneous fluorescence imaging and clear assessment of co-culture integrity.

Below are the staining procedure steps, with variations only in the specific antibody used and corresponding concentrations, adjusted according to each marker and sample type:

1. Cells cultured on CellDrum™ membranes were fixed using 1% paraformaldehyde for 10 minutes at room temperature, then washed three times with PBS to remove residual fixative.
2. Cells were permeabilized using 0.1% Triton X-100 in PBS for 5 minutes at room temperature, followed by three PBS washes. Samples were blocked with 5% FBS in PBS for 1 hour at room temperature.
3. Staining solutions were prepared using Alexa Fluor 647-conjugated CD31 (10 μ l in 500 μ l buffer) for endothelial cell identification and Alexa Fluor 488-conjugated α -SMA (1 μ l in 500 μ l buffer) for smooth muscle cell confirmation. Both antibodies were diluted in 5% FBS in PBS.
4. Samples were incubated with antibody solutions overnight at 4°C, then washed three times with PBS. Hoechst nuclear stain (1.5 μ l in 1498.5 μ l PBS) was applied for 10 minutes to visualize nuclei, followed by a final PBS wash before imaging.

2.6 Wound Healing Assay

Inspired by conventional stamping methods in in-vitro wound models, the mechanical properties of ultra-thin CellDrum™ membranes were considered when developing a suitable wound generation approach. Initially, scratch assays were tested but proved highly inconsistent, creating irregular wounding areas and occasionally ripping PDMS membranes.

Alternative methods including vacuuming and laser-based approaches were then evaluated. The vacuum method did not damage membranes but lacked sufficient suction to effectively detach

cells. Previous research indicated that 16 J/cm² laser dose reduced cell viability with significant membrane damage, but a 5-mW laser used on CellDrums showed no observable cell damage. Considering these limitations, stainless steel balls were ultimately adopted as this method proved better suited to the CellDrumTM system's mechanical characteristics while ensuring consistent and controlled wound generation^{111,112,140}.

2.6.1 Wounding Method

Stainless steel balls with a diameter of 2.5 mm (weighing 65.6 mg each) were sterilised by autoclaving and carefully positioned at the centre of the membrane surface using sterilised tweezers under optical guidance to ensure consistent placement. The CellDrums were then incubated with the steel balls in place for 48 hours, with medium changes performed according to standard laboratory protocols to maintain optimal culture conditions.

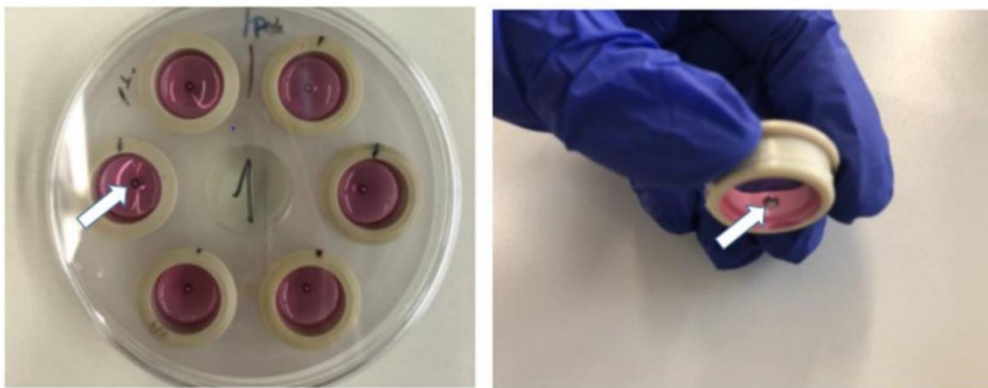


Figure 20: Representation of the 6-channel CellDrumTM holder used (left). Each CellDrumTM was filled with DMEM (pink-coloured), and the steel balls were placed (white arrows) in the middle of the membranes (right).

Due to the high elasticity and compliance of the CellDrumTM membrane, the steel ball weight applied localized, consistent pressure on the cell monolayer center, causing mechanical disruption of cells beneath the ball. This method effectively generated reproducible, mostly circular cell-free areas, simulating wound environments while avoiding variability and mechanical risks of manual scratching techniques. The mechanical damage also stimulated surrounding cells to initiate migratory activity towards the wound area. The cell-free region created by the stainless-steel ball is referred to as a "wound" and served as the basis for subsequent wound healing and mechanobiology experiments in this thesis.

2.6.2 Mechanical Stimulation of Dermal Fibroblasts via PulSelect

In total, 40 CellDrumTM membranes were seeded with dermal fibroblasts and grouped by donor age and mechanical training presence, creating 4 groups of 10 CellDrums each (control and training groups for both ages).

48 hours after wound creation, steel balls were removed, CellDrums were placed in airtight holders, and cells were exposed to cyclic strain at 1 Hz for 30 minutes followed by 2-hour rest. This cycle was repeated three times with media renewal between sessions. On the second day, CellDrums were stimulated once. This training protocol, though unpublished, has proven successful for cytoskeleton remodelling experiments in our laboratory.

To assess cell detachment from cyclic stretching, samples were examined under phase-contrast microscopy after each training session. Additionally, a previous unpublished study in our laboratory using live-dead cell imaging on the same cell lines with identical strain protocol showed that out of 200,000 seeded cells, approximately 514 were non-viable, corresponding only to 0.7% cell death. Based on these observations, the applied cyclic stretching protocol was considered appropriate as it did not negatively affect cell viability.

2.6.2 Fluorescence Staining of Dermal Fibroblasts

To improve image quality and cell visibility during mechanical stimulation experiments, cells were stained prior to training using CellTracker Green CMFDA dye. The staining procedure was performed according to the manufacturer's instructions, with a few optimisations for the CellDrum™ system:

1. The CellTracker vial was brought to room temperature. The lyophilised dye was dissolved in 10.75 μL of dimethyl sulfoxide (DMSO) to prepare a 10 mM stock solution.
2. The stock solution was diluted in serum-free medium to prepare a working solution within the recommended range of 0.5–25 μM .
3. Preliminary tests were conducted to determine the optimal working concentration for image quality and contrast, with 5 μM identified as the most suitable concentration for this setup.
4. Cell culture medium was aspirated from the CellDrums and replaced with the pre-warmed staining dye solution. The CellDrums were incubated with the dye for 30 minutes in a CO₂ incubator at 37°C.
5. Following incubation, the staining solution was aspirated and replaced with fresh culture medium to prepare the samples for mechanical stimulation and imaging.

The staining dye remains visible within the cultured cells for at least 72 hours under standard culture conditions, providing stable fluorescence signal without significant photobleaching. This stability allowed the monitoring of wound closure dynamics for up to four days following wound creation on the CellDrum™ membranes, enabling tracking of cell migration and proliferation.



Figure 21: KEYENCE Fluorescence Microscope with the custom CellDrum™ holder

2.6.3 Determination of Wound Size

Following wound creation using the steel ball method, initial wound size was determined as a reference point for closure analysis. After removing steel balls, fluorescence images of each wound were captured using the KEYENCE fluorescence microscope to visualize wound boundaries. Images were imported into ImageJ software, and wound areas were measured by manually outlining cell-free regions with the polygon selection tool to ensure precise edge marking.

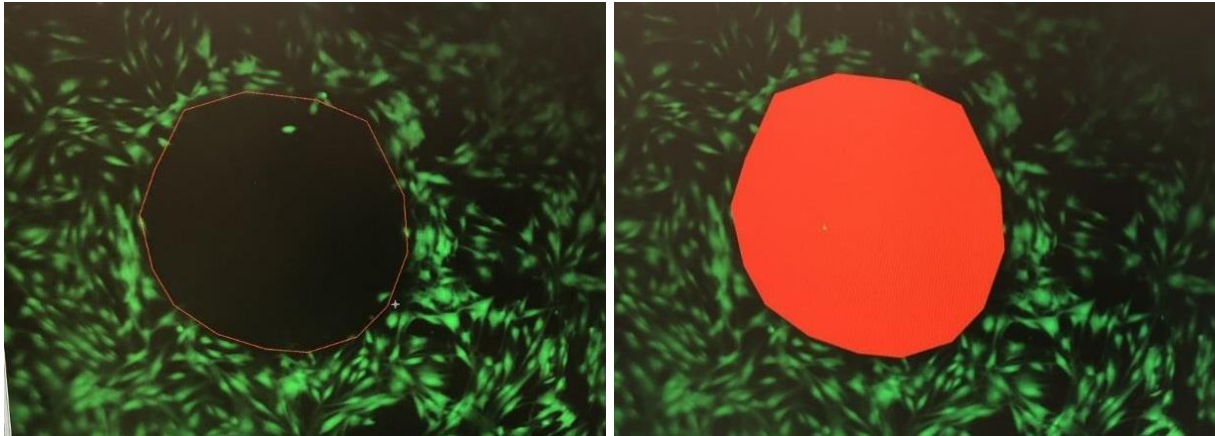


Figure 22: Outlining of the cell-free gap (R), measuring and calculation of the outlined area (L), Image J.

The calculated wound areas were recorded in square micrometres (μm^2) and saved for reference as the initial wound size ($Area_0$). These values served as the reference points for all subsequent time-point measurements and calculations of wound closure rates, providing consistency and reproducibility in the assessment of cell migration and gap closure during the wound healing assays.

2.6.4 Determination of Wound Closure Rate

Wound closure progress on CellDrumTM membranes was monitored using CellTracker dye and visualized with the KEYENCE fluorescence microscope. A custom 3D-printed holder positioned CellDrums securely during imaging for proper alignment and precise wound localization. Coordinates of each inspected area were recorded for consistent imaging of the same wound throughout all time points.

Images were captured at: (1) immediately after wound creation, (2) 7 hours after three mechanical stimulation sessions, (3) 11 hours, (4) 24 hours, (5) 48 hours, and (6) 96 hours post-stimulation. This schedule allowed detailed monitoring of wound healing, capturing immediate and longer-term cellular responses to mechanical stimulation. A total of 240 images were collected and analysed using the microscope's internal software. For each time point, wound area and circumference were measured using the polygon selection tool to manually outline the wound gap.

To quantify the wound closure rate, the Lundberg formula (Equation 3) (Lundberg et al., 1992) was utilized, using the wound area measurements from each time point in relation to the initial wound area ($Area_0$). In this calculation, $Area_i$ defines the wound area at the specific time point i , and the closure rate is expressed as the percentage of the remaining wound area relative to the initial wound size, thereby indirectly reflecting the extent of gap closure achieved over time. This approach allowed the systematic evaluation of cell migration and wound healing dynamics under defined mechanical and culture conditions used in these experiments.

$$\text{Wound closure rate (\%)} = \frac{Area_0 - Area_i}{Area_0} * 100 \quad (3)$$

2.7 Contractility Response Detection with CFA_{refer}

The cellular response to NA is complex due to the variability of adrenergic receptor subtypes across vascular cell types. This receptor diversity and associated downstream signalling pathways make it essential to carefully design experiments investigating NA effects on vascular cells. To evaluate NA effects on vascular cell monolayers and co-cultures, the CFA_{refer} device was employed for tension measurements on CellDrumTM membranes. In preliminary tests, HCAECs and HCASMCs were tested in monolayers and co-cultures to assess NA responsiveness.

As anticipated, HCAEC monolayers showed no significant membrane tension changes upon NA exposure and were excluded from subsequent testing. Following optimization of exposure times and NA concentrations, the main experimental series used HAOSMCs and HAOECs from ATCC.

For definitive measurements, only HAOSMC monolayers and HAOSMC+HAOEC co-cultures were tested for NA response to investigate contractile behaviour under conditions closer to in vivo vascular environments. To validate the measurement system, ionomycin calcium salt was employed as a positive control, inducing strong sustained contractions by increasing intracellular calcium levels and providing a reference for maximum contractile response. This approach allowed clear evaluation of NA mechanobiological effects on vascular cells cultured on CellDrumTM membranes.

2.7.1 Measurement Setup

CellDrums for NA response experiments were prepared as described in Section 2.2. Following membrane production, thickness was measured and only membranes within 3.2–4.0 μm were selected to ensure uniform mechanical properties. Membranes were visually inspected under transmitted light to exclude defects such as wrinkles or holes, then underwent autoclaving for sterility. Following chemical functionalization, intrinsic membrane tensions were measured for each CellDrumTM using 15 technical replicates with the CFA_{refer} system. Only membranes with stable, consistent tension values were retained to prevent abnormal stretching or non-uniform responses that could confound NA measurements.

After validation, membranes underwent a second autoclaving cycle before biological coating. 1% fibronectin solution was applied, and CellDrums were incubated at 4°C for 24–48 hours.

Prior to cell seeding, additional ultra-pure water was pipetted to pre-stretch membranes, ensuring consistent tension. Membranes were then left in a CO₂ incubator for 24 hours. Cell seeding was performed as described in Section 2.4.2. For HAOSMC monolayers, approximately 100,000 undifferentiated HAOSMCs were seeded onto fibronectin-coated CellDrums and maintained in growth medium for two days, then differentiated in differentiation medium renewed everyday over 8 days. For co-cultures, following 8-day HAOSMC differentiation, approximately 150,000 HAOECs were seeded onto HAOSMC monolayers and cultured in endothelial cell growth medium for two days prior to NA exposure.

2.7.2 Noradrenaline & Ionomycin Response on HAOSMC Monolayer

To systematically assess concentration-dependent effects of NA on vascular cell tension, measurements were conducted using the CFA_{refer} system on HAOSMC monolayer samples on CellDrumTM membranes. Five biological replicates were prepared, each measured in four technical repeats for data reliability. Technical repetitions were limited to four-five measurements to minimize risk of unintended mechanical responses from repeated pressure changes. The following procedure was followed for all NA measurements:

1. All measurements were conducted in 500 μ L differentiation medium. Control measurements using medium without NA established reference tension values before NA exposure.
2. To achieve desired NA concentrations (50-1000 nM), defined volumes of 1 μ g/mL NA working solution replaced equivalent medium volumes while maintaining 500 μ L total, with volumes ranging from 5-100 μ L corresponding to the respective concentrations.
3. After each NA addition, samples were incubated for 3 minutes before measurements. Measurements for each concentration were performed in four technical replicates. Medium was completely replaced with fresh differentiation medium before proceeding to next concentration. This process was repeated across all NA concentrations and biological replicates.

For contractile response determination, ionomycin calcium salt was used as positive control due to its ability to produce sustained intracellular calcium increases. Three biological replicates were assessed in four technical repeats using the following procedure:

1. All measurements were performed in 500 μ L differentiation medium with control measurements as baseline values. Tested concentrations were 1 nM, 10 nM, 100 nM, 1000 nM, and 10 μ M.
2. For the first three concentrations (1-100 nM), a 1 μ M working solution was prepared from 1 mM stock, with equivalent medium volumes replaced using 0.5-50 μ L. For higher concentrations (1000 nM and 10 μ M), 1 mM stock solution was used directly with 0.5-5 μ L added.
3. Measurements were conducted immediately after ionomycin addition without incubation to capture acute responses, with approximately 5 minutes recorded after addition. Medium

was replaced with fresh differentiation medium before adding the next concentration. This procedure was repeated until all concentrations were tested across all biological replicates.

2.7.3 Noradrenaline & Ionomycin Response on Vascular Co-culture

In the experiments investigating the effects of NA and ionomycin on co-culture (HAOSMC + HAOEC) samples, the same measurement procedures described above in Section 2.7.2 were used to maintain consistency and comparability with the monoculture experiments.

The only difference between the monoculture and co-culture trials was the number of biological replicates. For the NA measurements three samples were prepared while the sample size for ionomycin measurements consisted of two samples. All other experimental parameters, including medium volume, number of technical replicates, preparation of substance concentration, and exposure times were maintained as previously explained to ensure direct comparability across datasets.

2.7.4 Vitality Test

To confirm that observed effects during NA trials were due to active cellular responses and not artefacts from cell death or detachment, additional viability staining was performed in preliminary testing before and after NA exposure on HCASMC and HCAEC monocultures and co-cultures. Standard CellTracker staining was performed using CellTracker Red for HCASMCs and CellTracker Green for HCAECs), allowing clear visualization and identification within both systems. As these results confirmed cell viability under tested conditions, live-dead staining was not repeated in later NA trials.

2.8 Statistical Analysis

The following subsections present the statistical analysis methods used in each experimental trial that was performed in this thesis. The subsections are organized by experimental design and dataset, providing clear separation of methods to the corresponding experimental conditions. The selection of statistical tests was based on the distribution characteristics and sample sizes of the datasets, ensuring accurate analysis of the experimental results.

2.8.1 Membrane Optimization

The statistical analysis was performed using IBM's SPSS Statistical Analysis. Normality tests were performed for all groups with a Shapiro–Wilk test due to smaller group sizes ($n < 50$). For all experimental setups one-way ANOVA tests were performed. For groups that show overall significant differences, further post hoc tests were carried out (Tukey's HSD) to determine which exact groups differed between each other. The relationship between thickness and stiffness was statistically analysed by a linear regression test (Pearson's Function) after proving normal data distribution using IBM SPSS. The function gives values between “-1” to “1”, where both values indicate a strong correlation, either negatively or positively, while a “0” value shows no relationship between two parameters ¹⁴¹.

2.8.2 Wound Healing Assay

The statistical analysis was performed using GraphPad Prism. Each experimental group was checked for normal distribution. As the results showed non-normality, it was decided to continue with a non-parametric test. To compare the wound perimeter of each sample between different time points, a one-tailed (right) Wilcoxon-Signed-Rank test was chosen.

2.8.3 Noradrenaline Contractility Response

Statistical analysis of the noradrenaline and ionomycin trials was performed using GraphPad Prism. In the case of noradrenaline experiments on both HAOSMC monolayers and HAOSMC + HAOEC co-cultures, Friedman test with Dunn's post-hoc correction was used after verifying data normality, ensuring correct treatment of repeated measures within non-parametric datasets.

The ionomycin trials on HAOSMC monolayers were also evaluated using the same statistical approach after the data was tested for normal distribution. However, the ionomycin co-culture samples were analysed using a one-way repeated measures ANOVA with Holm–Šidák correction, as the dataset met the assumptions required for parametric testing. This selection of varying statistical methods guaranteed that each dataset was analysed using the most fitting approach based on the characteristics of data distribution, providing reliable comparisons between the tested conditions.

3. Results

3.1 Phase 1- CellDrum™ Optimization

3.1.1 Material Selection

To evaluate and compare the surface morphology and manufacturing quality of the attachment site of the CellDrum™ applicators, SEM analyses were conducted on PEEK and PP-HOMO materials.

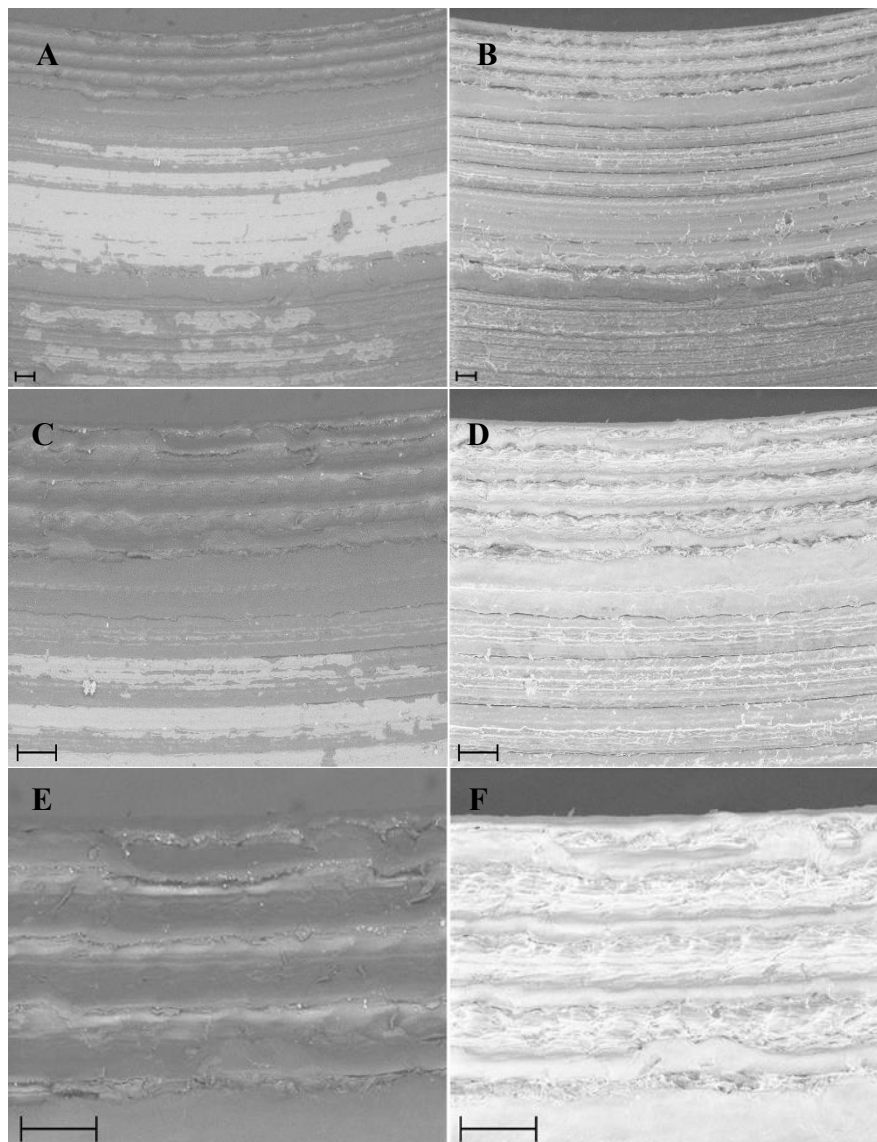
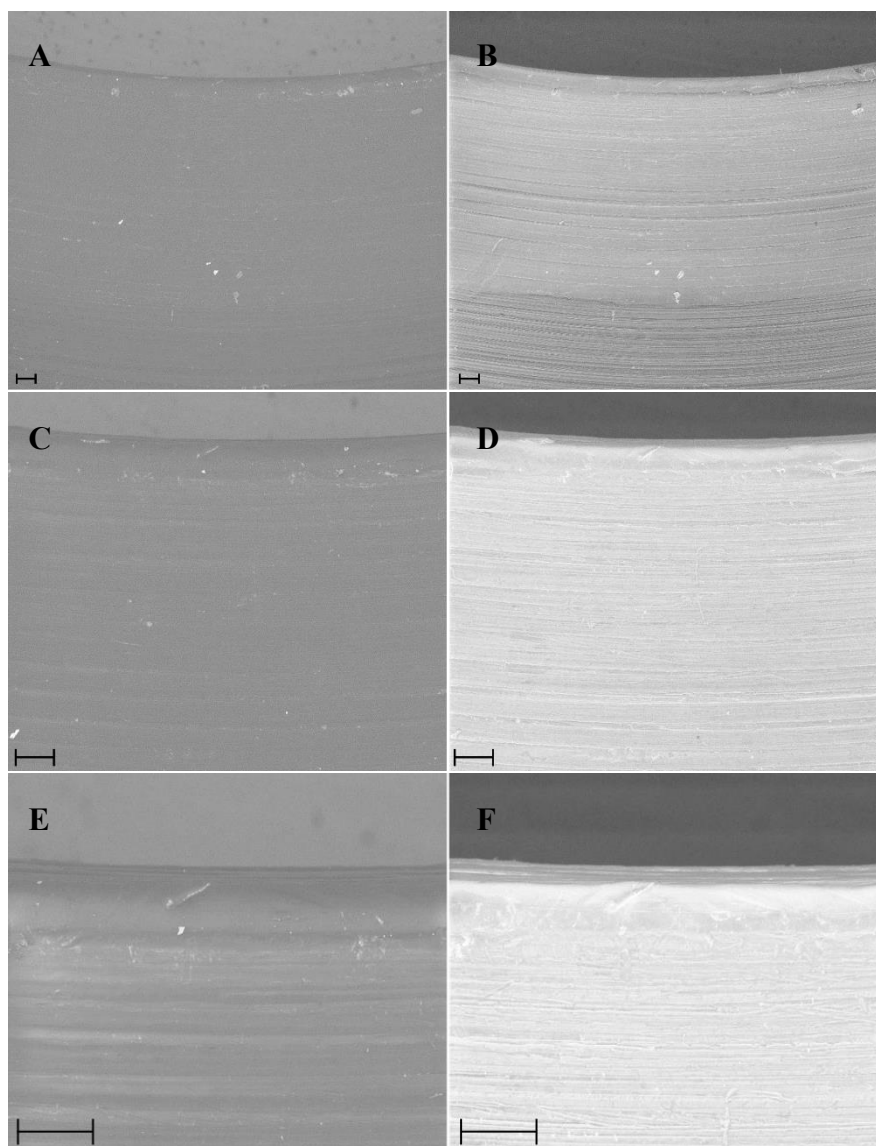


Figure 23: Top view SEM Images of PEEK material, A) 50x VPSE signal B) 50x BSD4-A signal, C) 100x VPSE signal, D) 100x BSD4-A signal, E) 200x VPSE signal and F) 200x BDS4-A signal. Scale bar: 100 μ m.

With the PEEK material, layered surface morphology characteristic of the machining-based production was clearly visible for all tested magnifications and with both signals, the circular fiber structure of the material was reflected. The VPSE signal provided a clearer visualization of surface heterogeneity, enabling the detection of topographical details, while the BSD4 A

signal emphasized compositional uniformity and highlighted deeper layers with higher contrast. At higher magnification (200x), surface impurities became significantly apparent, and it was observed in the VPSE image already at 50 $\times$ as well as in most BSD4 A images that certain areas of the surface were worn, recognizable by unevenly shaped and differently contrasted spots.



*Figure 24: Top view SEM Images of PP-HOMO material, **A)** 50x VPSE signal **B)** 50x BSD4-A signal, **C)** 100x VPSE signal, **D)** 100x BSD4-A signal, **E)** 200x VPSE signal and **F)** 200x BDS4-A signal. Scale bar: 100 μ m.*

Figure 24 demonstrates a visually clearer and smoother material surface for the PP-HOMO sample. At 50x magnification, both BSD and VPSE modes reveal a smooth and continuous surface with some visible surface irregularities and scattered particulate matter. The edges appear well-defined, and no obvious structural defects or delamination are visible.

At 100x magnification, finer surface texture becomes more apparent, especially in the VPSE image. Parallel patterns are visible, likely related to the manufacturing process. The BSD image continues to show a homogeneous surface with few embedded particulates. Finally, at 200x magnification, both detector modes provide enhanced visualization of the polymer layering and fine surface features. The VPSE image especially highlights the layered structure with clearer

horizontal orientation. The BSD image also shows fine surface particulates and maintains edge integrity. The scale bars indicate that surface features are consistent in size and structure across the field of view, with no signs of major discontinuities or structural inhomogeneities.

3.1.2 Biocompatibility of CellDrum Applicator Materials

In the search for a cost-effective alternative to PEEK for CellDrum applicators, potential materials were first compared on paper with respect to autoclavability, thermal and mechanical stability, and production feasibility. Based on these criteria, PP-HOMO was identified as the most suitable candidate, offering durability and resistance to repeated sterilization without deformation. Following this selection, the material was subjected to biocompatibility testing with fibroblasts to assess its suitability within the experimental setup. Biocompatibility of the CellDrum Applicator material was evaluated based on fibroblast proliferation and cell vitality.

To test the suitability of PP-HOMO for CellDrum™ applicators, biocompatibility was evaluated with human dermal fibroblasts under standardized conditions. Two experimental groups were used: wells containing either 2 or 4 sterilized PP-HOMO pieces (2M and 4M). The hollow pieces were small enough to fit into 6-well plates and light enough to float on the culture medium without pressing on the cell layer. By varying the number of PP-HOMO pieces, the total material surface area in contact with the culture differed, enabling evaluation of potential dose-dependent effects on cell viability and proliferation. This provided key data for the potential use of PP-HOMO in CellDrum™ production and in vitro mechanobiological studies.

Table 4: Control group results for both 2 and 4 day tests, N=6

Biocompatibility Test, Control Group (N=6)				
Duration	Average Cell Count	Average Live Cell Count	Vitality	SEM
2 days	452035	434890	96.2%	0.43%
4 days	651360	626890	96.2%	0.98%

Table 5: Experiment group results for both 2 and 4 days, N=3

Biocompatibility Test, Experimental Group (N=3)					
Duration	Material	Average Cell Count	Average Live Cell Count	Vitality	SEM
2 days	2 M	328445	320220	97.5%	0.88%
2 days	4 M	243083	234750	96.6%	1.08%
4 days	2 M	627665	616500	98.2%	0.39%
4 days	4 M	616665	609780	98.9%	0.33%

In the control group (Table 4), cell numbers increased ~1.4-fold between day 2 and day 4, corresponding to a growth rate of 20% per day, while vitality remained at 96.2%. In the experimental groups (Table 5), proliferation was higher, with ~1.9-fold (38%/day) and ~2.5-fold (59%/day) increases in the 2M and 4M groups, respectively. Vitality values were consistently high across all groups (96.2-98.9%), and low SEM values confirmed reproducibility.

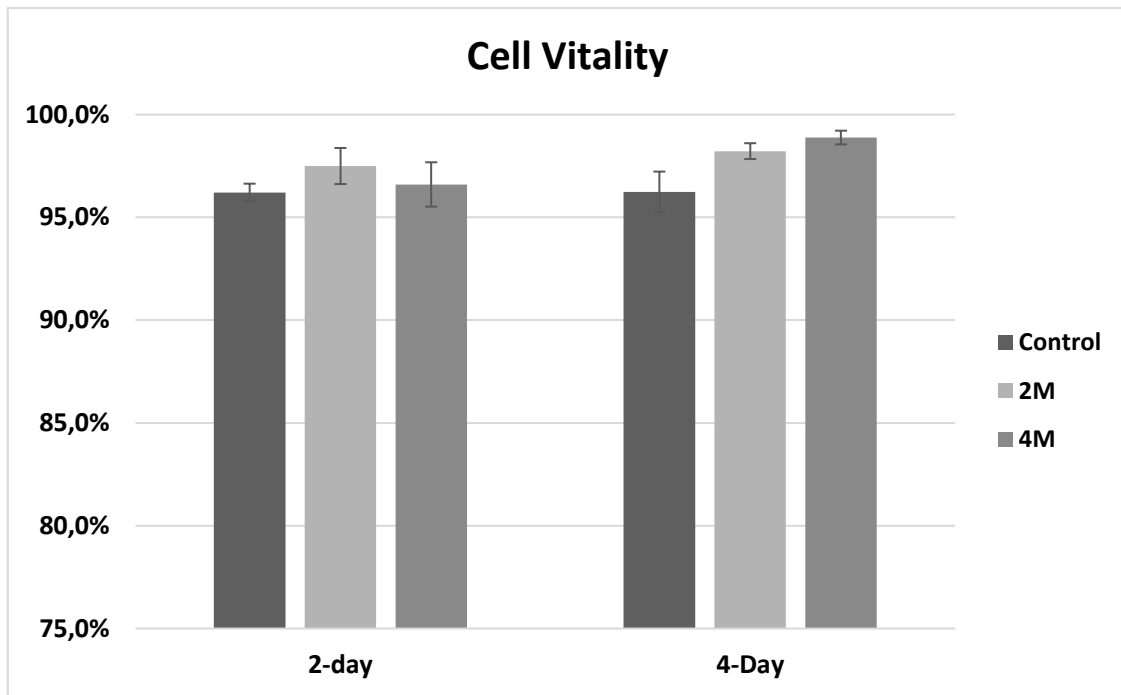


Figure 25: Quantitative evaluation of cell vitality (%) in control, 2M, and 4M groups after 2-day and 4-day cultivation periods.

A one-way ANOVA with Tukey HSD correction was used to compare the vitalities of the groups (Figure 25). The ANOVA significance analysis showed no statistically significant difference between the vitalities of the control, 2M, and 4M groups at a 2-day duration ($p > 0,05$). Specifically, the p-value for the difference between the control and 2M was 0.5886, between the control and 4M was 0.8425, and between 2M and 4M was 0.9002.

Due to a violation of the assumption of normality, a non-parametric Kruskal-Wallis test was conducted to compare the 4-day exposure effects of the control, 2M, and 4M groups. This test, followed by Dunn's comparisons with a Sidak correction, revealed no statistically significant differences between the groups. Specifically, the pairwise comparisons showed that the p-value for the control and 4M group was 0.04834, which did not meet the adjusted significance threshold of 0.01695. Similarly, no significant differences were found between the control and 2M group ($p = 0.4107$), or between the 2M and 4M group ($p = 0.2494$). Overall, these results show that the PP-HOMO material does not have any negative effect on the health of the cell culture and can be considered biocompatible.

3.2 Evaluation and Optimization of Membrane Production Parameters

3.2.1 Dependency of Membrane Thickness on PDMS Volume

Since its introduction and patenting, CellDrum™ technology has been continuously optimized and experimentally adapted, particularly by J. Trzewik^{17,18}. This work further developed the CellDrum production process into a standard operating procedure, with special focus on membrane fabrication, to ensure consistent future results.

During fabrication, PDMS was pipetted onto the water surface where it spread out and became briefly visible under reflected light before it turned nearly transparent within seconds. To ensure uniform membrane thickness, the distance between the PDMS drop at the pipette tip and the water surface was minimized to prevent bouncing or uneven distribution of the elastomer. All experiments were conducted in the same room to minimize environmental variability. The weak bonding between PDMS and the water surface facilitated easy transfer of cured membranes onto CellDrum™ applicators as free-standing films. Three different PDMS volumes were tested for membrane production: 4, 8, and 10 μL

Table 6: Measured membrane thickness values at various PDMS volumes. Numbers in parentheses represent the “calculated” values for the chosen volumes.

PDMS Volume (μL)	Average Thickness (μm)	Standard Deviation (μm)
4.00	3.47	0.23
8.00	7.49 (6.94) ¹	0.25
10.00	9.43 (8.68) ²	0.63

The 4 μL group (Group 1) served as baseline with a mean thickness of $3.47 \pm 0.23 \mu\text{m}$ ($n = 11$). Doubling the volume to 8 μL (Group 2) yielded $7.49 \pm 0.26 \mu\text{m}$, representing a 116% increase versus the theoretical 100% expected. Group 3 (10 μL) achieved $9.43 \pm 0.63 \mu\text{m}$ thickness. The measured fold increases were 1.72 (Groups 1-3) and 0.26 (Groups 2-3), compared to theoretical values of 1.50 and 0.25 respectively. While Groups 2-3 matched expectations perfectly, the 1-3 comparison showed consistently thicker membranes than predicted, likely indicating non-linear volume-thickness relationships.

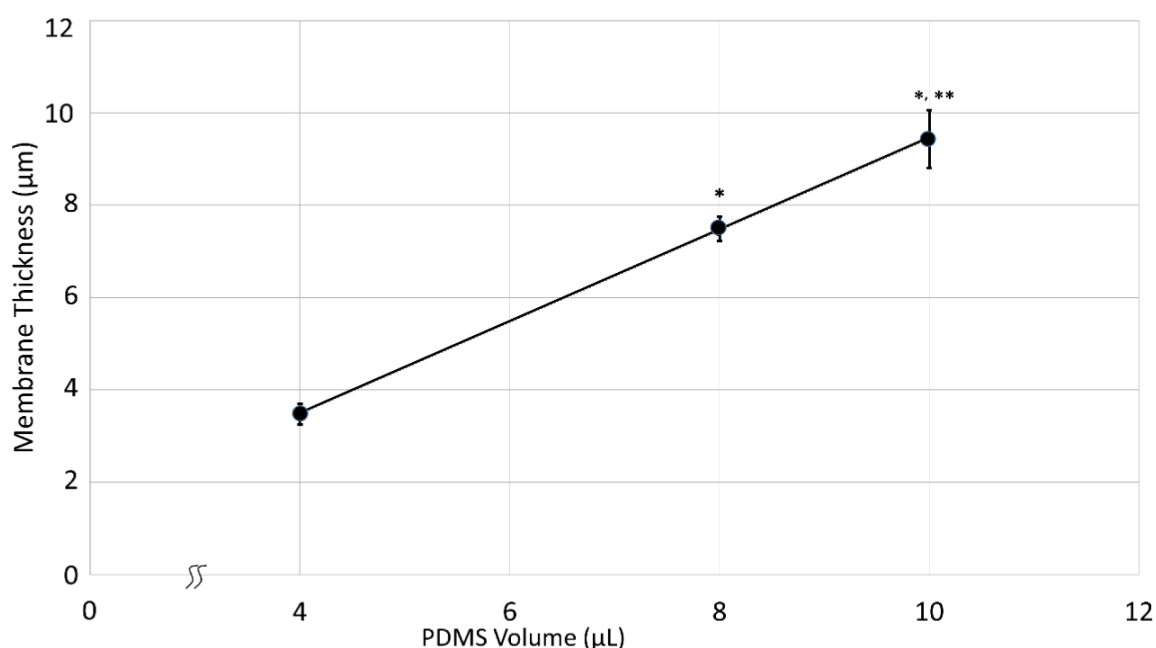


Figure 26: PDMS membrane thickness as a function of PDMS volume. Averages and standard deviations of all groups ($n = 11$ per group) are shown including the regression line.

All three groups were then compared using one-way ANOVA, which showed significant differences between each group within a 99% confidence interval (* $p < 0.01$ vs. 4 μL group, ** $p < 0.01$ vs. 8 μL group; $r^2 = 0.97$), confirming non-coincidental results (Figure 26). The temperature during production was kept within ± 0.5 $^{\circ}\text{C}$, as temperature deviations result in PDMS viscosity changes causing thickness variations between groups. Therefore, temperature effects were tested next, with results presented in section 3.2.2.

3.2.2 Dependency of Membrane Thickness on room temperature

The following investigation of membrane thickness was conducted using a constant PDMS volume of 4.15 μL while varying the ambient room temperature during PDMS elastomer spreading on the water surface. The selected temperatures of 22, 24, and 26 $^{\circ}\text{C}$ represent typical laboratory environment conditions encountered across different seasons. Temperature control was maintained consistently using the same methods for each trial, with no variation occurring during the fabrication process. Group sizes were identical for all groups, $n = 14$. All three batches tested positive for normal distribution before the one-way ANOVA test was performed.

Table 7: Measured thickness values of membranes produced in different room temperatures, $n=14$

Temperature ($^{\circ}\text{C}$)	Average Thickness (μm)	Standard Deviation (μm)
22	4.00	0.40
24	3.72	0.30
26	3.68	0.35

Table 7 and Figure 27 both show the effect of ambient temperature on membrane thickness during fabrication. At 22 $^{\circ}\text{C}$, the membranes exhibited the highest average thickness of 4.00 μm ($\text{SD} \pm 0.40$ μm). As temperature increased to 24 $^{\circ}\text{C}$ and 26 $^{\circ}\text{C}$, the average thickness decreased to 3.72 μm ($\text{SD} \pm 0.30$ μm) and 3.68 μm ($\text{SD} \pm 0.35$ μm), respectively.

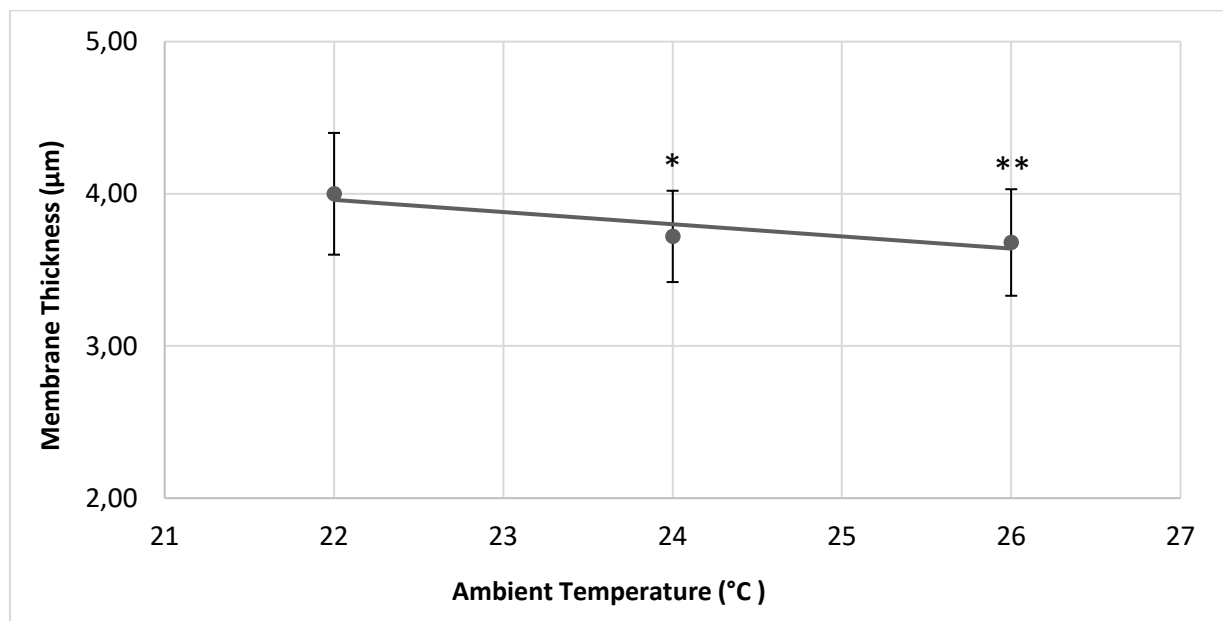


Figure 27: Membrane thickness as function of the environmental temperature.

One-way ANOVA showed significant differences between groups 1–2 and 1–3, but not between 2–3 (* $p < 0.05$ vs. Group 1, ** $p < 0.01$ vs. Group 1, $r^2 = 0.83$). The trend illustrates a mild but consistent inverse relationship between ambient temperature and membrane thickness.

3.2.3 Membrane Functionalization

To ensure reliable use of the CellDrum™ system in cell-based experiments, surface functionalization of the PDMS membrane was carried out to promote most of all cell attachment but also affect the biocompatibility. Functionalization was considered a success when cells adhered uniformly, remained viable over time, and responded appropriately under mechanical and pharmacological conditions. Various cell types were tested to evaluate the robustness of the coating procedure, including vascular smooth muscle cells, endothelial cells, dermal fibroblasts, and cardiomyocytes. Below are representative examples of some of the cell types tested on the functionalized CellDrum™ membranes. These were selected based on their biological relevance and importance to the aims of this thesis, particularly in the context of vascular and connective tissue research.

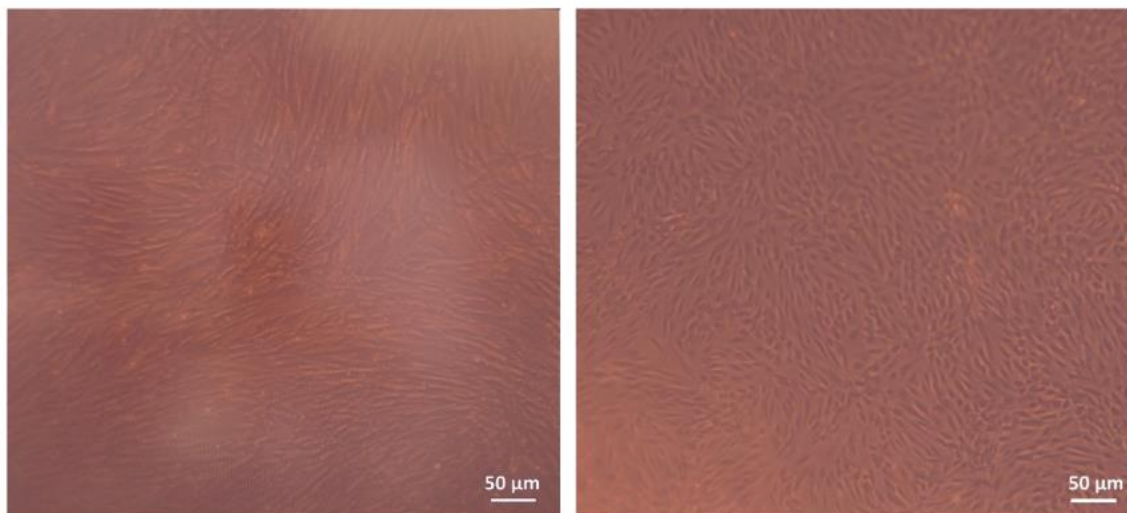


Figure 28: Phase contrast images of cell monolayers on CellDrum™ PDMS membranes, (left) Human arterial smooth muscle cells (hASMC), and (right) Human Dermal Fibroblasts-Adult (nHDFa).

Figure 28 shows representative phase-contrast images of two different cell types cultured on the functionalized CellDrum™ membrane. Differentiated human arterial smooth muscle cells (haSMCs) and human dermal fibroblasts (nHDFa) display healthy, elongated morphologies and uniform distribution. Both cell types appear well-adhered and form confluent monolayers, indicating successful attachment and compatibility with the membrane surface. Throughout the experimental procedures conducted in this study, and in previous studies from the same laboratory involving CellDrum™-based cultures, no significant cell detachment was observed. This remained consistent even under extended cultivation conditions lasting up to 14 days

3.2.4 Interdependency of Membrane Thickness and Deflection

To investigate the interdependence between membrane thickness and stiffness, a two-part experimental approach was employed. The first set of experiments focused on assessing the relationship between membrane thickness and passive membrane deflection under static conditions. In a complementary second approach, the connection between membrane thickness and mechanical parameters, specifically membrane compliance (referred to as membrane tension in the Noradrenaline trials), was examined using the CFA_{refer} measurement system.

Even though the fabrication and quality control procedures of the CellDrum membranes were standardized, due to the manual production, minimal parameter fluctuations were to expect. Membrane transfer via the mechanical aid onto the CellDrumTM applicator provides the membrane with an initial pretension within the membrane, that cannot be manually regulated. Different pretensions on membranes lead to different deflection values despite using the same load atop. For membrane deflection measurements, two test setups were used. To induce deflection, 500 μ L PBS hydrostatic load was applied in both setups. In the first trial, the deflection of the membranes was measured from a batch of CellDrums ($n = 35$) that were prepared simultaneously under the same environmental conditions. Thicknesses of these membranes ranged between 3.28–3.98 μ m.

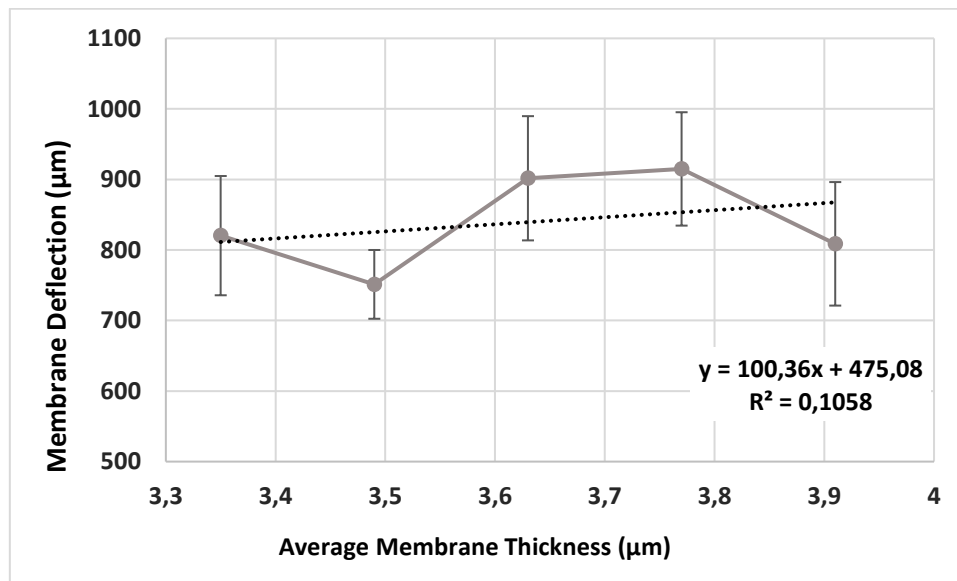


Figure 29: Dependency of membrane deflection vs. membrane thickness within a range of 3.28–3.98 μ m. For each thickness group, the average thickness value was taken, error bars represent standard error of mean (SEM).

Table 8: Measured average deflection values of membranes with different thickness ranges

Membrane Thickness (μ m)	Membrane Deflection (μ m)	Standard Error of Mean (μ m)
3.28–3.42 (Avg. 3.35)	820.33	84.54
3.42–3.56 (Avg. 3.49)	751.22	48.76
3.56–3.70 (Avg. 3.63)	901.67	88.07
3.70–3.84 (Avg. 3.77)	914.92	80.36
3.84–3.98 (Avg. 3.91)	808.73	87.56

Within the investigated thickness range of 3.28–3.98 μm , no significant correlation was identified, as indicated by a Pearson correlation coefficient of 0.10. This absence of correlation may be attributed to the relatively narrow distribution of thickness values. An R^2 value of 0.1 further supports the lack of dependence between the membrane thickness values in the given range to the membrane deflection results. Nonetheless, the selected range represents the most frequently used membrane thickness interval for classic, 16mm CellDrum™ formats. A one-way ANOVA test further proved the similarity and comparability of the data with a non-significant result at $\alpha = 0.05$ (Figure 29, Table 8).

The second test series concerning membrane deflection was carried out with the membranes that were shown above in Table 6. The deflection measurements of the varying PDMS volume groups (4, 8 and 10 μL) with $n = 11$ were repeated three-times and compared using a one-way ANOVA test. Almost a doubling of the deflection values was seen between 4- and 8 μL as well as 4- and 10 μL volume groups, which can be further visualized in Figure 30.

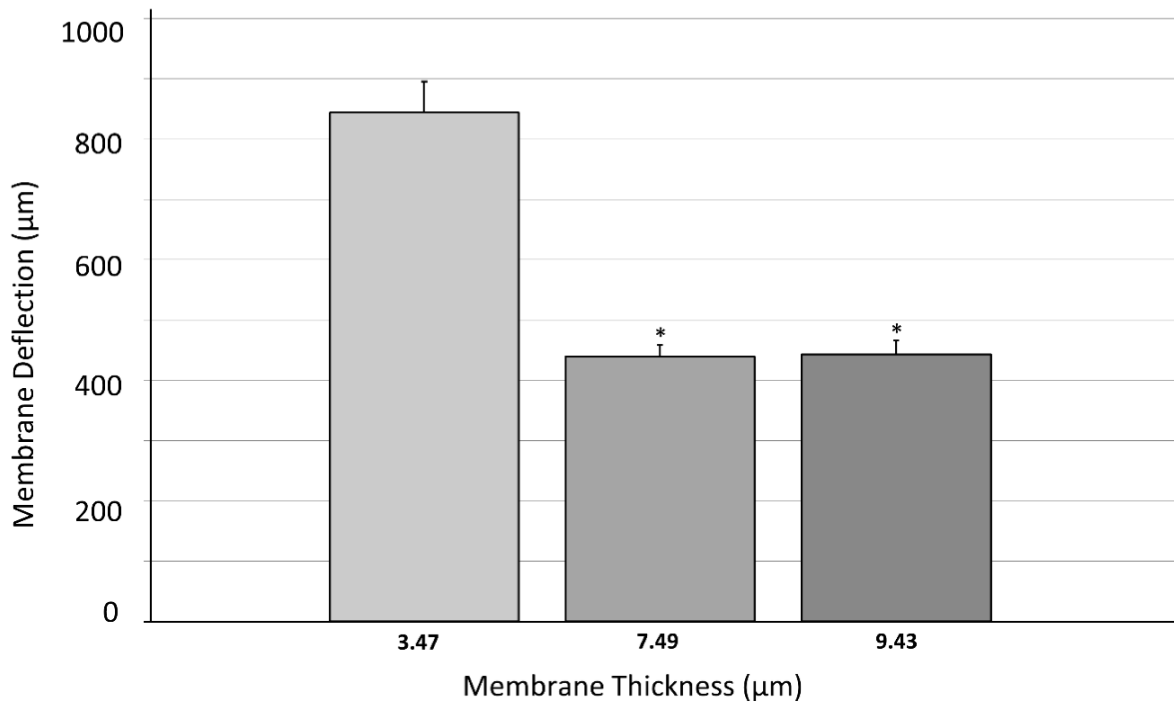


Figure 30: Dependency of membrane deflection on PDMS membrane thickness (thickness range = 3.24–10.06 μm). Error bars represent standard error of mean (SEM) for each group.

While group 1 showed an average deflection of 844 ± 52 μm , groups 2 and 3 revealed deflection values of 439 ± 19 μm and 443 ± 24 μm respectively (Table 11). One-way ANOVA showed significant differences between groups 1–2 and 1–3 (4 and 8 μL and 4 and 10 μL PDMS volume groups) with $\alpha = 0.01$ (* $p < 0.01$ vs. Group 1). However, groups 2–3 (8–10 μL PDMS volume) showed no significant difference, which could be due to the smaller volume and thickness difference between the membranes of each group compared to group 1.

Table 9: Measured average deflection values of membranes with average membrane thicknesses $\pm$ standard deviation in μm .

Membrane Thickness (μm)	Membrane Deflection (μm)	Standard Error of Mean (μm)
3.47 ± 0.23	844.1	51.53
7.49 ± 0.25	439.27	19.2
9.43 ± 0.63	442.64	23.74

The one-way ANOVA test showed no significant difference between the five thickness groups that were shown in the first deflection trial (Figure 29). However, as the thickness range was increased, highly significant differences were revealed between different thickness groups at $\alpha = 0.01$ (Figure 30).

To demonstrate the need for rigorous quality control procedures, an additional analysis was performed examining mechanical variability under identical production conditions. Five membranes with identical thicknesses were selected from the first deflection trial (Table 8) and their average deflection values were separately compared. Despite having nearly identical average thickness of $3.648 \pm 0.004 \mu\text{m}$, these membranes showed substantial deflection variability: $916 \mu\text{m}$ (1), $1378 \mu\text{m}$ (2), $939 \mu\text{m}$ (3), $569 \mu\text{m}$ (4), and $1008 \mu\text{m}$ (5). This lack of correlation between thickness and deflection highlights how initial membrane pre-tension and other factors can significantly affect mechanical characteristics, emphasizing the critical importance of post-production quality assessment.

3.2.5 Interdependency of Membrane Thickness and Compliance

To overcome possible systemic errors from the deflection trials (Section 3.2.4), a different test setup was used. A sample group of 30 CellDrums was investigated for their compliance in the $\text{CFA}_{\text{refer}}$ measurement device. The results were separated into four thickness groups with seven to nine samples each, with the help of a histogram.

The groups average compliance value was calculated (Table 10). A Shapiro–Wilk test proved that all populations were normally distributed, so a one-way ANOVA test could be performed. The test showed no significant difference between the groups, when using a significance level of $\alpha = 0.05$ (Figure 31).

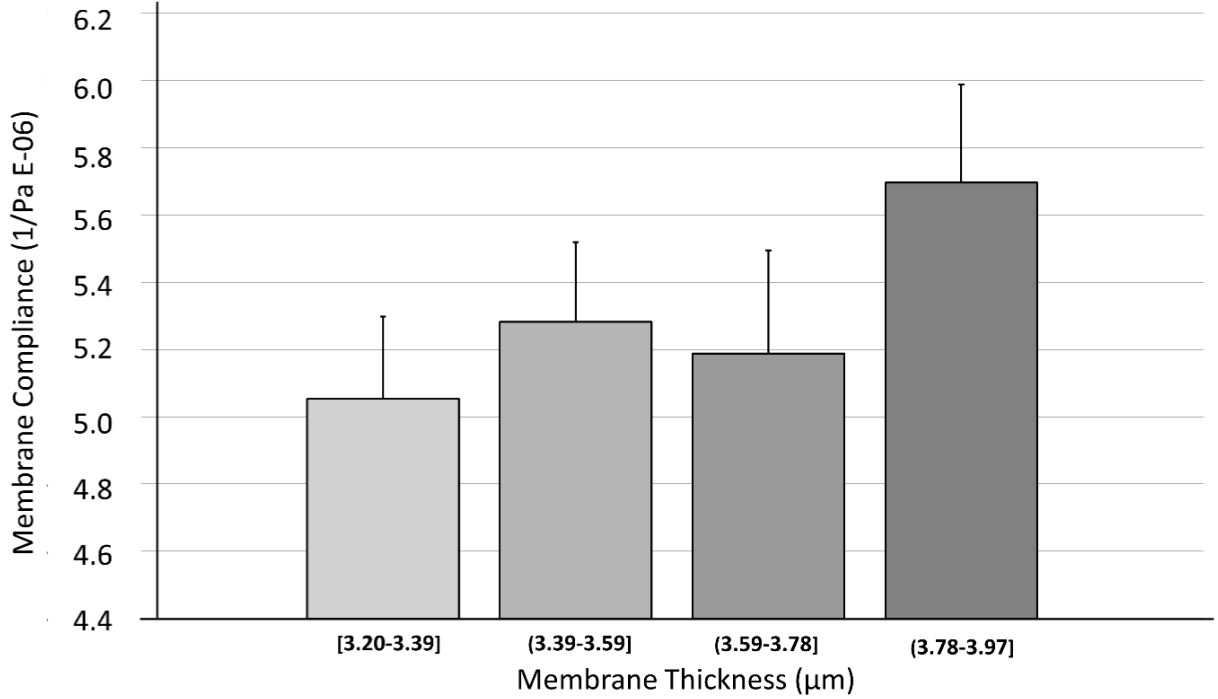


Figure 31: Dependency of membrane compliance on PDMS membrane thickness. Error bars represent standard error of mean (SEM) for each group.

Figure 31 shows membrane compliance values across four defined membrane thickness intervals ranging from 3.20 to 3.97 μm . The lowest compliance was observed in the thinnest group [3.20–3.39 μm], with an average of approximately 5.05×10^{-6} 1/Pa. Compliance values slightly increased in the [3.39–3.59 μm] and [3.59–3.78 μm] groups, reaching around 5.3 and 5.2×10^{-6} 1/Pa, respectively. The highest compliance was observed in the thickest group (3.78–3.97 μm], averaging about 5.7×10^{-6} 1/Pa. Despite these trends, statistical analysis using one-way ANOVA revealed no significant differences between the groups ($\alpha = 0.05$).

Table 10: Measured compliance values of membranes with different thickness ranges.

Membrane Thickness (μm)	Membrane Compliance (1/Pa E-06)	SEM (1/Pa E-06)
[3.20–3.39]	5.05	0.25
(3.39–3.59]	5.28	0.24
(3.59–3.78]	5.19	0.31
(3.78–3.97]	5.70	0.29

Table 10 shows the results presented in Figure 31, with the SEM values of the average membrane compliance for each tested membrane thickness group. Once again, we can see that there is no strictly proportional relationship between membrane thickness and membrane compliance, as the second highest value of membrane compliance can be found in one of the thinnest membrane groups.

3.3 Phase 2- Wound Healing Assay

3.3.1 CellDrum™ Membranes used in this assay

A total of 40 membranes were selected for further experimentation following visual pre-selection, quality control, and thickness measurements (see Table 11).

Table 11: Descriptive statistics for membrane thickness values for all sample groups, with SEM (Standard Error of Mean). The numbers 28 and 88 stand for the age of the donor which the dermal fibroblast cells were harvested from. C = Control Group and T = Training Group.

Membrane Thickness in μm				
Experimental Groups	Average	SEM	Total Average	Total SEM
<i>n=10</i>	28 T	3.70	3.69	0.04
	28 C	3.69		
	88 T	3.66		
	88 C	3.70		

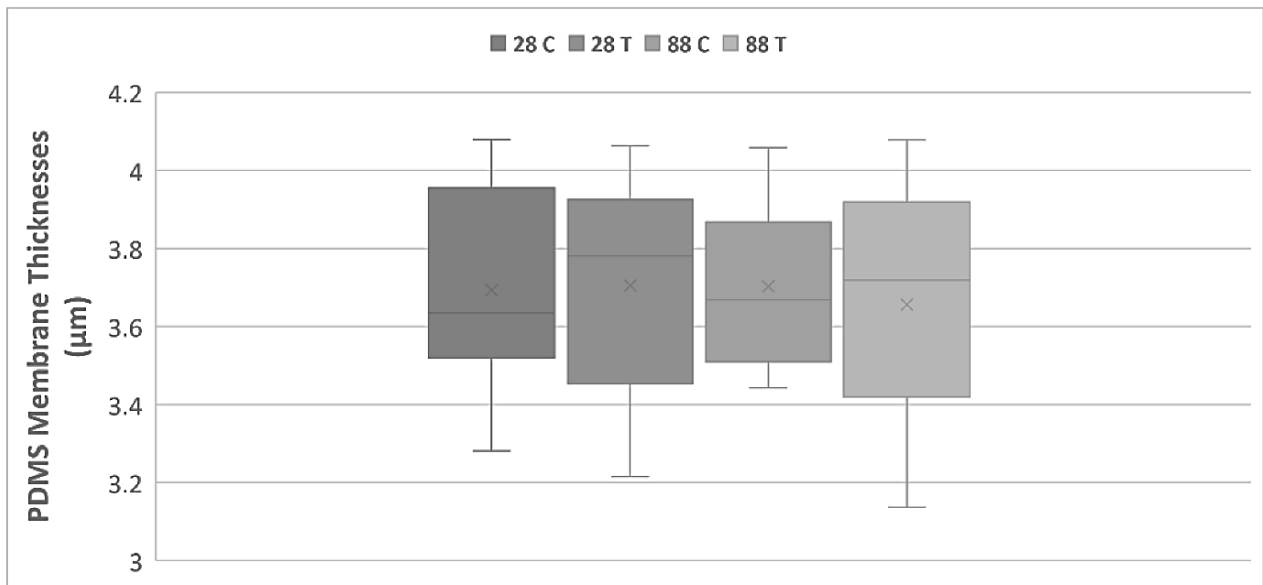


Figure 32: PDMS membrane thickness (μm) distribution for all experimental groups. The numbers 28 and 88 stand for the age of the donor which the dermal fibroblast cells were harvested from. C = Control Group and T = Training Group. The lower and upper fences are 25th and 75th percentiles, while the middle line represents the median, and the letter X stands for the sample average. The lower and upper whiskers show the minimum and maximum sample values, respectively.

Overall, thickness distributions appear highly consistent between control (C) and training (T) groups across both age groups as seen in Figure 32.

3.3.2 Gap Perimeter Standardization

To assess the uniformity of the created cell free “wounding” area by the steel ball technique, the initial cell free “wound” perimeter was chosen as the investigated parameter (Figure 33).

The figure shows the initial wound perimeters of the four experimental groups. The 28 T group displayed the largest perimeter ranging from approximately 580 to over 1000 μm . Median values across groups ranged between about 620 and 750 μm , with the 88 T group showing the lowest median but the broadest overall range (between ~ 400 to 1000 μm). Average wound sizes were slightly higher in the 28 T and 88 T groups compared to their respective controls.

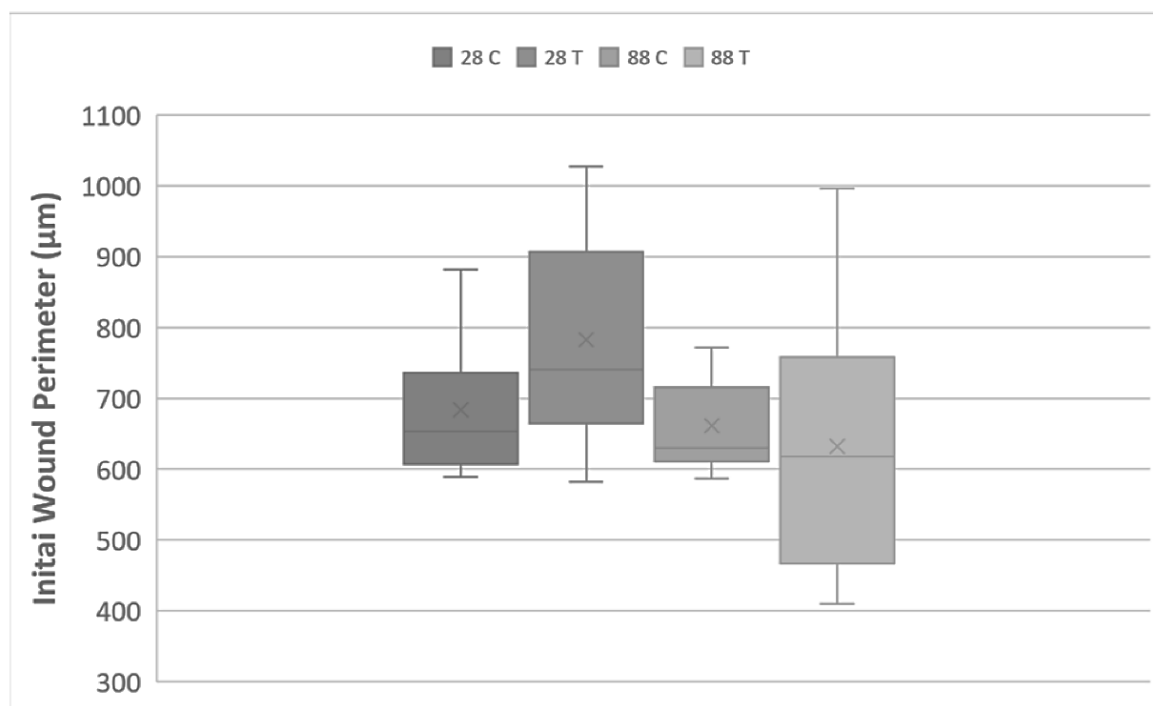


Figure 33: Boxplot of the initial wound perimeter for all samples (in μm). The lower and upper fences are 25th and 75th percentiles, while the middle line represents the median, and the letter X stands for the group average. The lower and upper whiskers show the minimum and maximum sample values, respectively. The numbers 28 and 88 indicates the donors age which the dermal fibroblast cells were harvested from. C = Control Group and T = Training Group.

Two outliers were identified and excluded from subsequent calculations: one from the 28-year-old control group (perimeter: 1112.13 μm) and another from the 28-year-old training group (perimeter: 1190 μm). These deviations occurred due to steel ball movement during sample transportation, as the flexible CellDrumTM membrane surface normally provides stable positioning, unlike rigid surfaces such as silicon or plastic culture flasks where unintended displacement creates larger, inconsistent wound gaps. Following outlier removal, wound areas ranged from 410 μm to 1027.45 μm . Initial wound perimeters showed the following group means: 28-year-old control group at 684.7 ± 35.1 μm , 28-year-old training group at 779.3 ± 47.7 μm , 88-year-old control group at 661.2 ± 19.4 μm , and 88-year-old training group at 632.1 ± 54.4 μm , with an overall mean of 687.02 μm . Since no significant differences existed between initial wound perimeters of older and younger donors, these membranes were deemed suitable for the wound healing assay experiments.

3.3.3 Gap Perimeter Change as Function of Wound Closure

Images illustrating the progression of wound closure for each group are presented in Figures 34 through 37, while the corresponding average perimeter values are summarized in Figure 38. Since the gaps did not uniformly close in a circular pattern, the evaluation of gap perimeter values was performed using a Polygon tool as explained in Section 2.6.3.

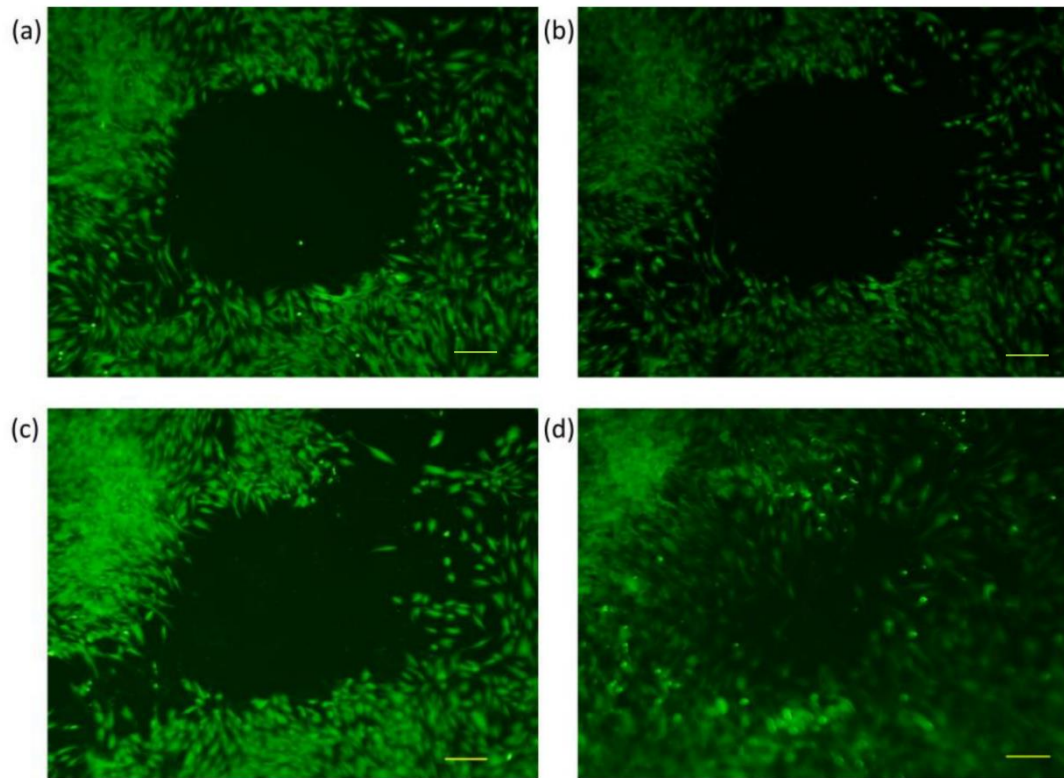


Figure 34: Fluorescence microscopy images of dermal fibroblasts from a 28-year-old donor at four different wound closure observation times (Control Group) (a) Initial, (b) 24 h, (c) 48 h, and (d) 4 days. Scale bar represents 50 μm .

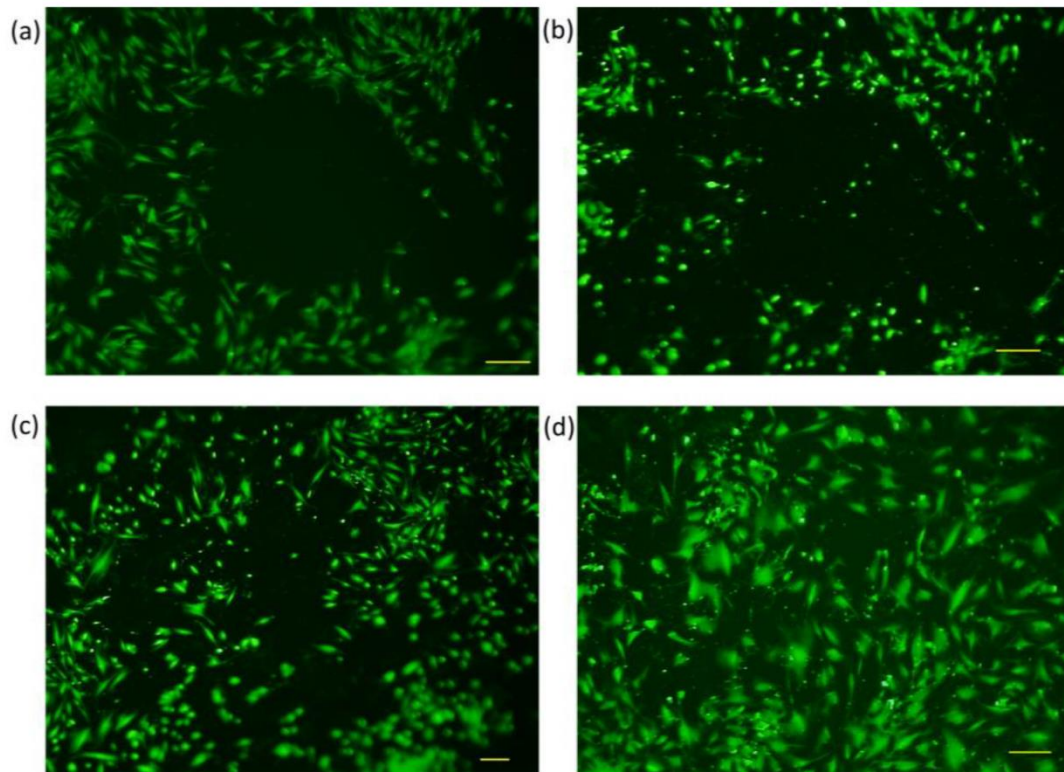


Figure 35: Fluorescence microscopy images of dermal fibroblasts from a 28-year-old donor at four different wound closure observation times (Training Group) (a) Initial, (b) 24 h, (c) 48 h, and (d) 4 days. Scale bar represents 50 μ m.

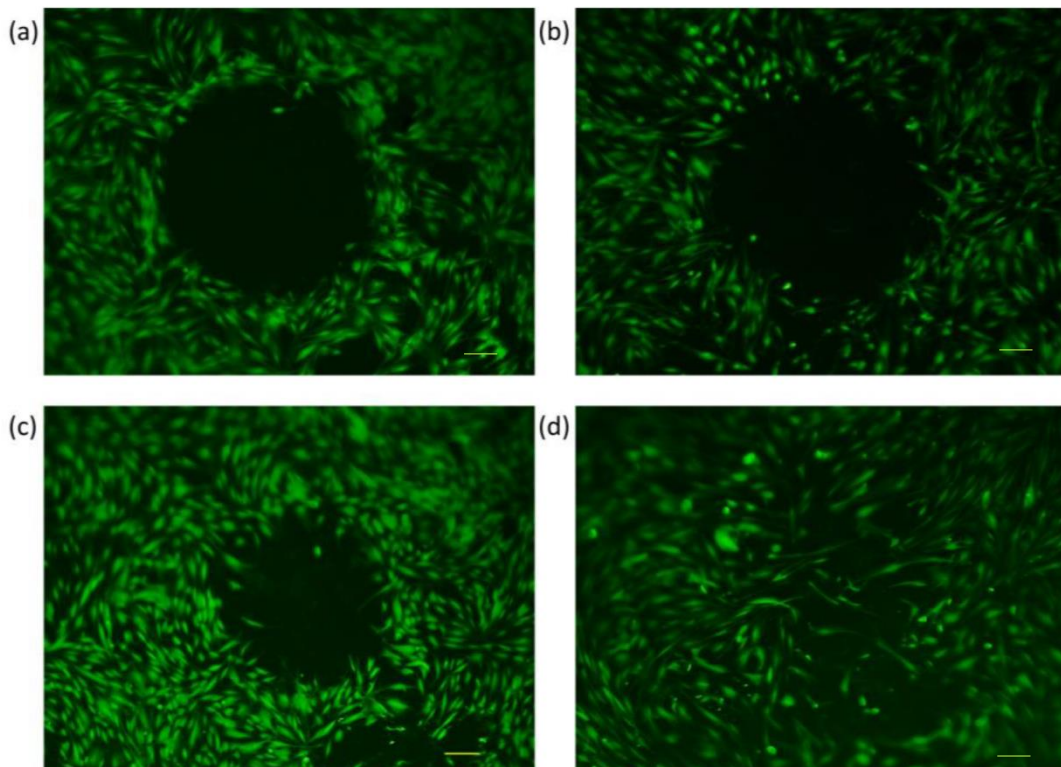


Figure 36: Fluorescence microscopy images of dermal fibroblasts from an 88-year-old donor at four different wound closure observation times (Control Group) (a) Initial, (b) 24 h, (c) 48 h, and (d) 4 days. Scale bar represents 50 μ m.

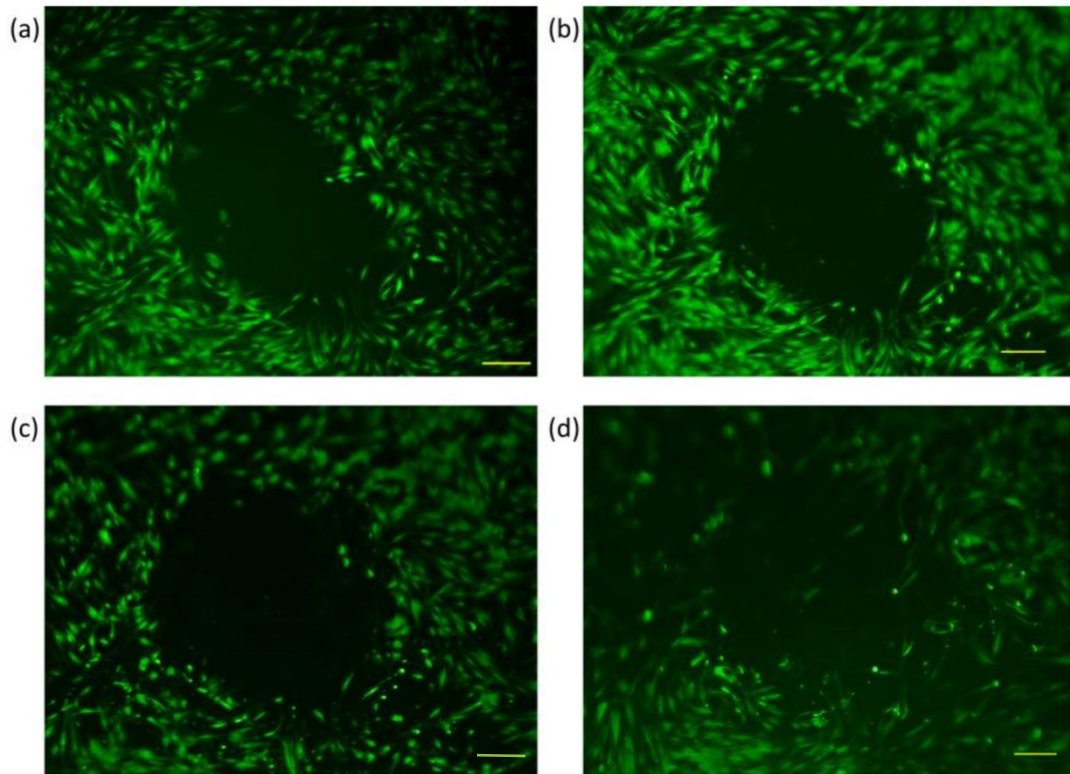


Figure 37: Fluorescence microscopy image of dermal fibroblasts from an 88-year-old donor at four different wound closure observation times (Training Group) (a) Initial, (b) 24 h, (c) 48 h, and (d) 4 days. Scale bar represents 50 μm .

Fluorescence images captured on day 4 appear noticeably less defined compared to earlier time points. This reduced clarity is attributed to the declining stability and intensity of the CellTracker dye, as its fluorescence signal diminishes over time. Such signal degradation is a known limitation of cell-permeant dyes and is consistent with the expected photostability profile, particularly beyond 72 hours post-labelling.

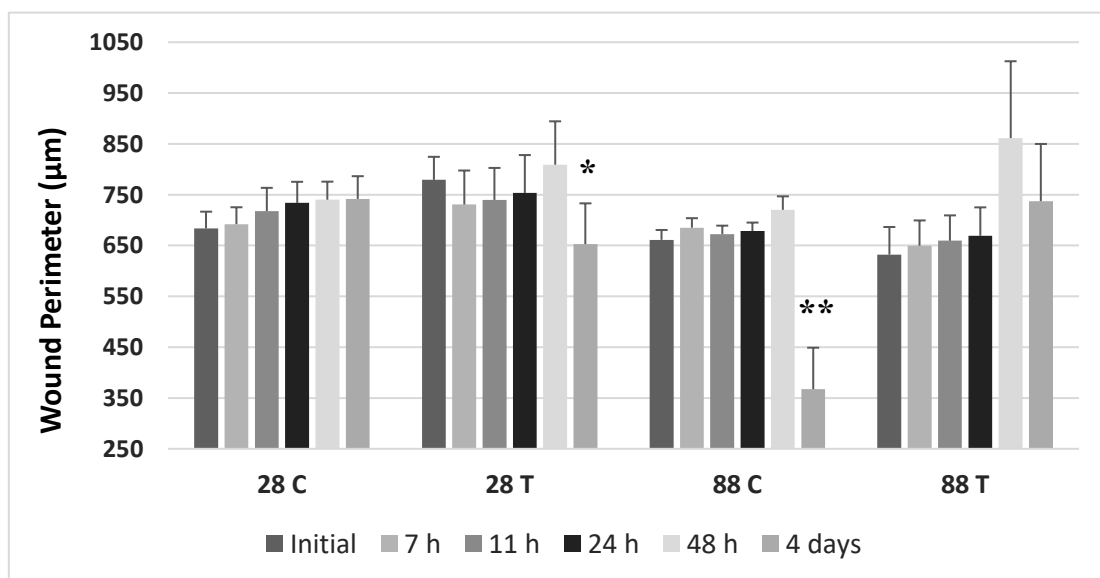


Figure 38: Cell training induced change of wound perimeter compared to non-trained cells (mean $\pm$ SEM) (* $p < 0.05$, ** $p < 0.01$ significance compared to initial time point).

Table 12: Average wound perimeter $\pm$ SEM in μm for dermal fibroblasts of 28- and 88-year-old donors, $n=10$. (* $p < 0.05$, ** $p < 0.01$ significance compared to initial).

Average Wound Perimeter (μm)						
Group	Initial	7 h	11 h	24 h	48 h	4 days
28 C	683.7 $\pm$ 32.9	692.0 $\pm$ 33.3	717.7 $\pm$ 45.8	733.8 $\pm$ 41.6	740.1 $\pm$ 35.6	741.7 $\pm$ 44.7
28 T	779.3 $\pm$ 45.2	730.6 $\pm$ 67.0	739.7 $\pm$ 63.0	753.4 $\pm$ 74.6	809.1 $\pm$ 85.3	652.8 $\pm$ 80.2 *
88 C	661.2 $\pm$ 19.4	685.1 $\pm$ 18.7	672.3 $\pm$ 16.8	678.6 $\pm$ 16.6	720.2 $\pm$ 26.6	367.4 $\pm$ 81.6 **
88 T	632.1 $\pm$ 54.4	649.6 $\pm$ 49.8	659.4 $\pm$ 49.9	669.2 $\pm$ 55.9	861.4 $\pm$ 151.2	737.2 $\pm$ 112.6

As seen in Table 12 and Figures 34–37, there is a seemingly contradicting pattern between the control and training groups of dermal fibroblast cells from 88-year-old donors vs. 28-year-old ones. The wound perimeter appeared to decrease after mechanical stimulation for the young donor, while cells from the 88-year-old donor showed an increase in wound perimeter with stimulation at the end of 4 days. Table 12 and Figure 38 further illustrate the changes in the average wound perimeter across all samples. From these changes in the perimeter values, the gap-closure rate was calculated (see Section 2.6.4) to assess and compare the effects of aging and mechanical stimulation on wound healing, with the results summarized in Table 13.

Table 13: Vectoral gap closure rates of all sample groups. Negative values indicate an increase in gap perimeter and vice versa (* $p < 0.05$, ** $p < 0.01$ significance compared to initial)

Gap Closure Rate (%)				
Experimental Groups		24 h	48 h	4 days
<i>n=10</i>	28 C	-10	-27	-10
	28 T	6	1	33 *
	88 C	-3	-17	55 **
	88 T	-14	-111	-49

In the control group of the younger donor samples, no significant change in wound closure was observed over the 4-day period, with a gap-closure rate of -10% , which can be considered negligible. In contrast, the application of mechanical training had a significant positive effect ($p < 0.05$) on wound closure after 4 days. The gap-closure rate in the trained group reached 33% , indicating a marked improvement in wound healing under external mechanical stimulation (for stimulation parameters, see Section 2.2.3) These findings suggest that mechanical training promotes enhanced wound closure in this experimental group. One-way ANOVA ($p = 0.0007$) indicated differences among the four groups, and post-hoc analysis identified which groups differed significantly.

Table 14: Post-hoc analysis of the gap closure rate

Treatment Pairs	Tukey HSD p-value	Significance
trained 28 vs control 28	0.56	Insignificant normal “n.s.”
trained 28 vs trained 88	0.001	** $p < 0.01$
trained 28 vs control 88	0.899	insignificant
control 28 vs trained 88	0.118	insignificant
control 28 vs control 88	0.602	insignificant
trained 88 vs control 88	0.002	** $p < 0.01$

For dermal fibroblasts derived from the 88-year-old donor, opposing trends were observed in the wound closure dynamics between the control and trained groups. A highly significant reduction in the average wound perimeter was measured in the control group after 4 days ($p < 0.01$), corresponding to a gap-closure rate of 55%. In contrast, the trained group exhibited a significant increase in wound size over the same period, resulting in a negative gap-closure rate of -49%.

3.4 Phase 3- Cellular tensile stress measurement with the CFA_{refer}

In earlier measurements, the CFA_{refer} device reported membrane mechanics as compliance (1/Pa E-06) when both the device and software were in prototype stages. By Phase 3, the finalized device outputs values directly as membrane tension in mN/mm². This change improves clarity, as "compliance" has varying interpretations across disciplines like materials science and cellular biomechanics and also eliminates rounding errors from multi-step calculations required to derive compliance from raw measurements.

3.4.1 CellDrumTM Membranes used for mechanical testing experiments

CellDrums are physical sensors that are assigned to a unique ID number. Therefore, each individual CellDrum used must be manufactured according to strict standardised laboratory procedures (Section 2.3). The functionality of the CellDrum must also be thoroughly tested before use via a strict quality control procedure. The quality control included visual inspection, thickness measurements, and baseline tension testing with 15 technical replicates. This testing also served as pre-stretching to identify and exclude mechanically unstable membranes before biological functionalization for optimal cell adhesion. Membranes with only less than 2% variation in their baseline tension measurements were taken into further experimentation.

Table 15: Overview of the CellDrums selected for noradrenaline and exposure experiments using HAOSMCs monolayers. The table includes CellDrumTM ID and membrane thickness

Noradrenaline HAOSMC Monolayer, n=5		Ionomycin HAOSMC Monolayer, n=4	
CellDrum TM ID	Thickness (μm)	CellDrum TM ID	Thickness (μm)
CD1	3.71	CD6	3.56
CD2	3.83	CD7	3.56
CD3	3.56	CD8	3.84
CD4	3.71	CD9	4.00
CD5	3.57		

Table 16: Overview of the CellDrums selected for noradrenaline and ionomycin exposure experiments using HAOSMC+HAOEC co-cultures. The table includes CellDrumTM ID and membrane thickness.

Noradrenaline Co-Culture, n=4		Ionomycin Co-Culture, n=2	
CellDrum TM ID	Thickness (μm)	CellDrum TM ID	Thickness (μm)
CD10	3.68	CD14	3.83
CD11	3.56	CD15	3.38
CD12	3.54		
CD13	3.72		

3.4.2 Phase Contrast Imaging of Cell Morphology

Throughout the course of the experiments, phase contrast microscopy was routinely employed to visually monitor the condition and morphology of the cultured cells at key time points. Observations were conducted before seeding, two days post-seeding (HAOSMC monolayer and HAOEC co-culture), after the differentiation phase and following exposure to noradrenaline. At each of these stages, representative images were captured to document cellular confluency, attachment, and morphology, ensuring consistency and quality throughout the experimental procedures.

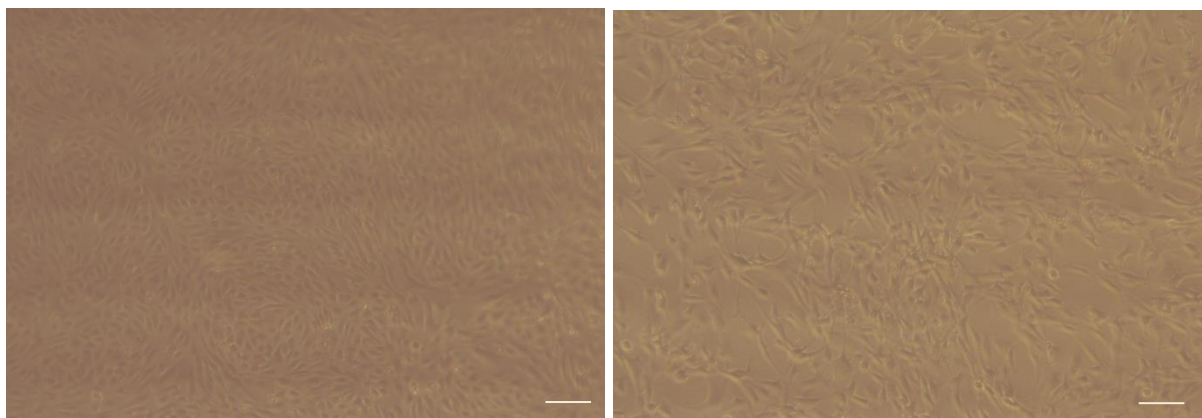


Figure 39: Phase contrast microscopy images (5X) of HAOECs (left) and HAOSMCs (right) in standard culture flasks prior to seeding on CellDrum™ membranes. Scale bar represents 50 μ m.

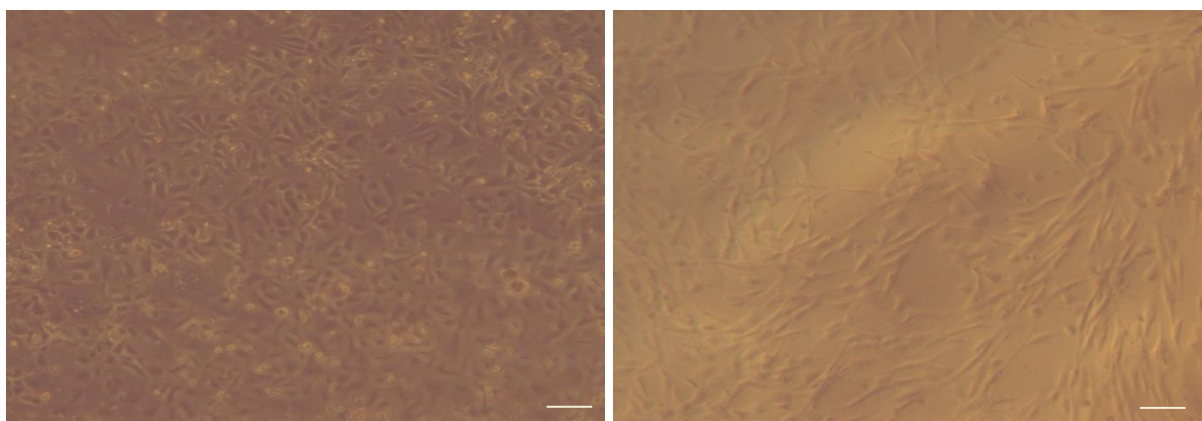


Figure 40: Phase contrast microscopy images (5X) of HAOECs (left) and HAOSMCs (right) on CellDrum™ membranes 2 days after seeding.

In Figure 40, phase contrast microscopy images of HAOECs and HAOSMCs cultured individually on CellDrum™ membranes taken two days after seeding can be seen. The HAOECs exhibited a typical cobblestone-like morphology, indicative of healthy endothelial monolayers. The HAOSMCs displayed an undifferentiated, spread-out morphology characteristic of proliferative smooth muscle cells. These observations suggest that both cell types were able to adhere to and proliferate on the CellDrum™ membranes under standard culture conditions, supporting the membrane's suitability for vascular cell monolayers.

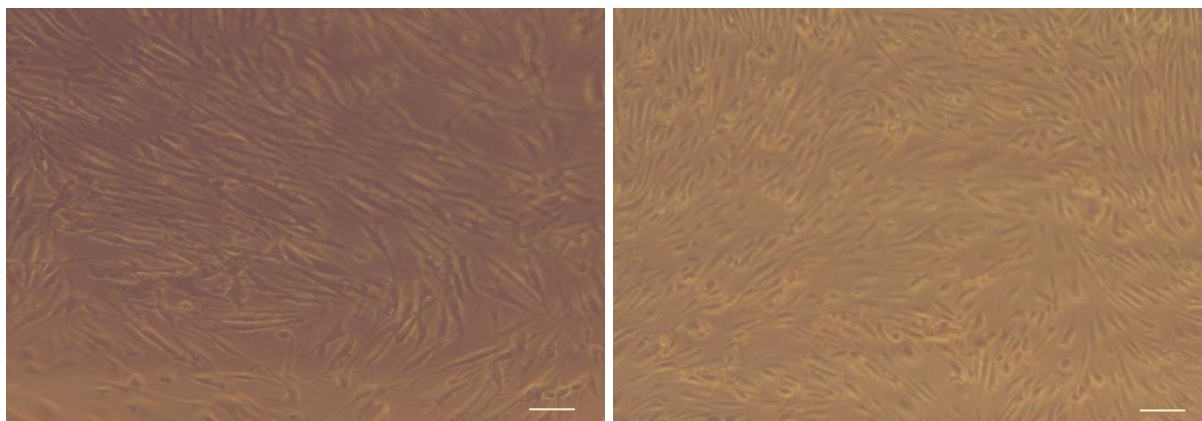


Figure 41: Phase contrast microscopy images (5X) of HAOSMCs-differentiated (left) and HAOSMCs + HAOECs co-culture (right) in CellDrums 2 days after seeding.

In Figure 41, phase contrast microscopy images can be seen two days after seeding as they display HAOSMCs in their differentiated, contractile phenotype on the left, and a co-culture of HAOSMCs and HAOECs on the right, both cultivated on CellDrum™ membranes. The HAOSMCs on the left exhibit the typical elongated, spindle-shaped morphology associated with a contractile state, indicating successful induction of differentiation. On the right, the co-culture shows a heterogeneous cell population with distinguishable morphological features of both smooth muscle and endothelial cells. These observations confirm the viability and morphological integrity of both monolayer and co-culture conditions on the CellDrum™ membranes.

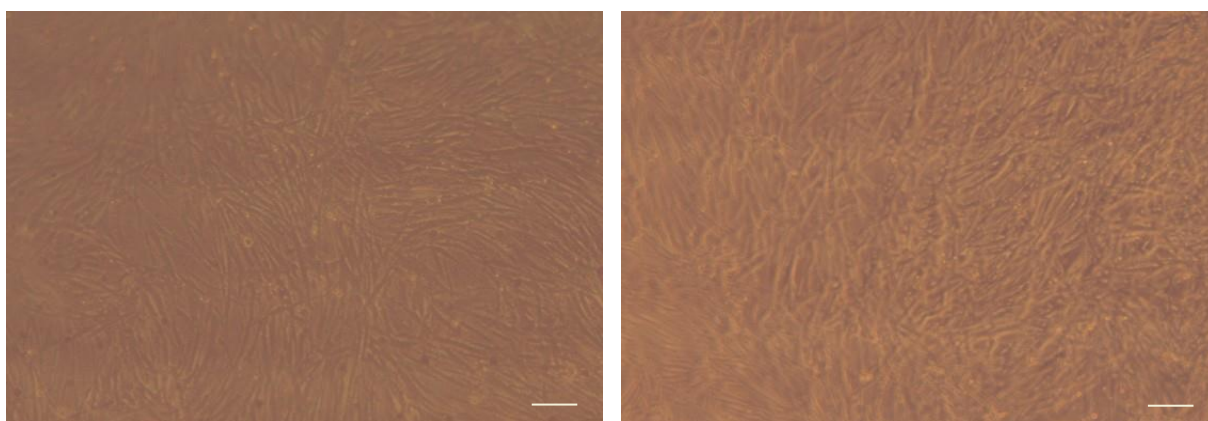


Figure 42: Phase contrast microscopy images (5X) of HAOSMCs Monolayers before- (left) and after 1000 nM NA exposure (right) in CellDrums.

The phase contrast microscopy images presented in Figure 42 show HAOSMC monolayers on CellDrum™ membranes before (left) and after (right) exposure to 1000 nM noradrenaline (NA). No obvious morphological alterations were observed following substance exposure, indicating that the cells preserved their structural integrity and remained viable

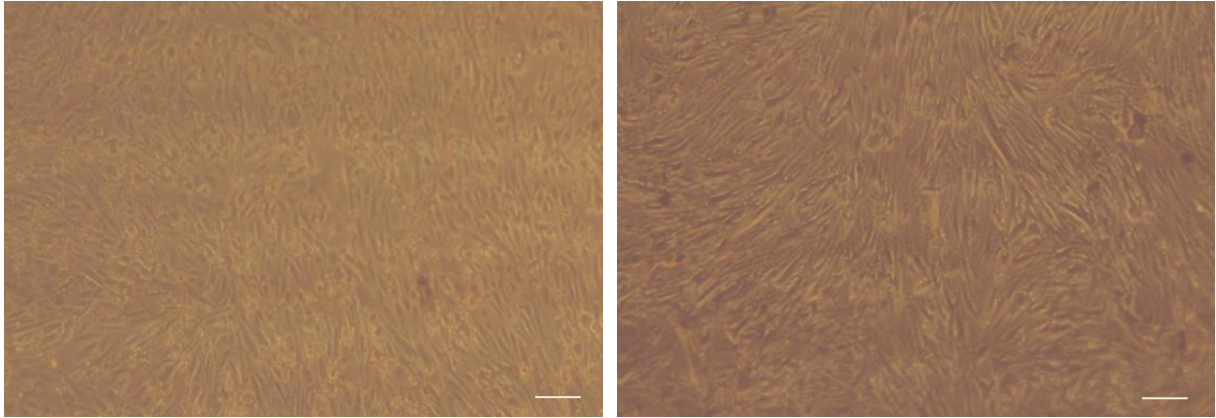


Figure 43: Phase contrast microscopy images (5X) of HAOSMC+HAOEC co-culture before (left) and after 1000 nM NA exposure (right) in CellDrums.

In Figure 43, phase contrast microscopy images presented can be seen of HAOSMC+HAOEC co-cultures on CellDrum™ membranes before and after exposure to 1000 nM NA. Morphological assessment revealed no significant structural changes in the cells following stimulation. A slight change in cell direction was observed, likely due to membrane movement during tension measurements when the air pump caused repeated stretching and relaxation. The monolayer remained stable and confluent, with both cell types maintaining viability and characteristic morphology.

3.4.3 Vitality Test

In addition to phase contrast imaging, cell vitality in both individual cultures and co-cultures was assessed using CellTracker dyes, which selectively stain viable cells. Different dye colours distinguished cell types and confirmed their distribution across the membranes. HAOSMCs were labelled with CellTracker Red and HAOECs with CellTracker Green in both monolayer and co-culture conditions. These dyes provided distinct fluorescence signals for cell-type identification and served as a basic viability check. This initial labelling confirmed successful seeding and co-culture establishment on the CellDrum™ membranes before proceeding with cell-specific fluorescence markers.

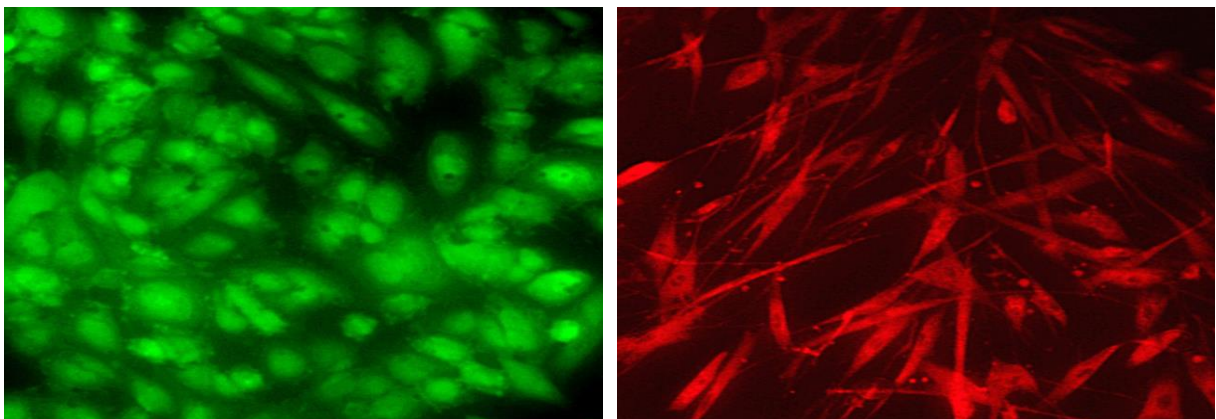


Figure 44: KEYENCE Fluorescence microscopy images (20X) showing monolayers of HAOECs (left) labelled with CellTracker™ Green and HAOSMCs (right) labelled with CellTracker™ Red.

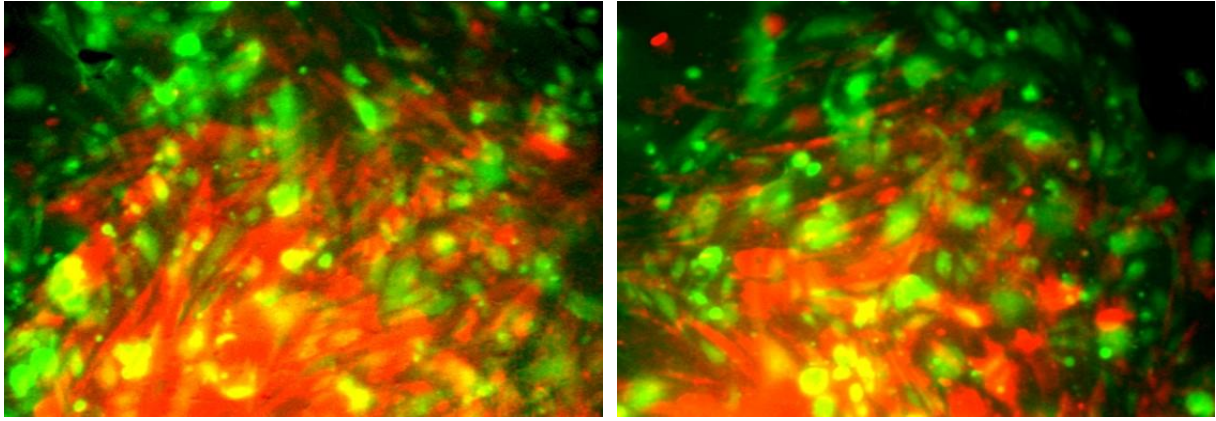


Figure 45: KEYENCE Fluorescence microscopy images (20X) showing **co-cultures** of HAOECs labelled with CellTracker™ Green and HAOSMCs labelled with CellTracker™ Red.

3.4.4 Fluorescence-Based Characterization by Immunostaining

In order to confirm the identity and phenotype of the cell types used in the experiments, immunofluorescence staining was conducted on monolayer cultures of HAOSMCs and HAOECs, as well as on their co-cultures.

HAOSMCs were stained with Alexa Fluor 488-conjugated anti- α -SMA antibody, confirming their identity and contractile phenotype. HAOECs, used as a negative control, showed no α -SMA signal. Additionally, HAOECs stained with Alexa Fluor 647-conjugated anti-CD31 confirmed their endothelial identity, while HAOSMCs showed no CD31 expression, validating marker specificity. Following validation in monolayers, a co-culture of HAOSMCs and HAOECs was stained using both α -SMA (Alexa Fluor 488) and CD31 (Alexa Fluor 647), with an additional nuclei staining using Hoechst stain. The following confocal microscopic images were taken with the kind assistance of Dr. habil. Tomo Šarić, at the University of Cologne.

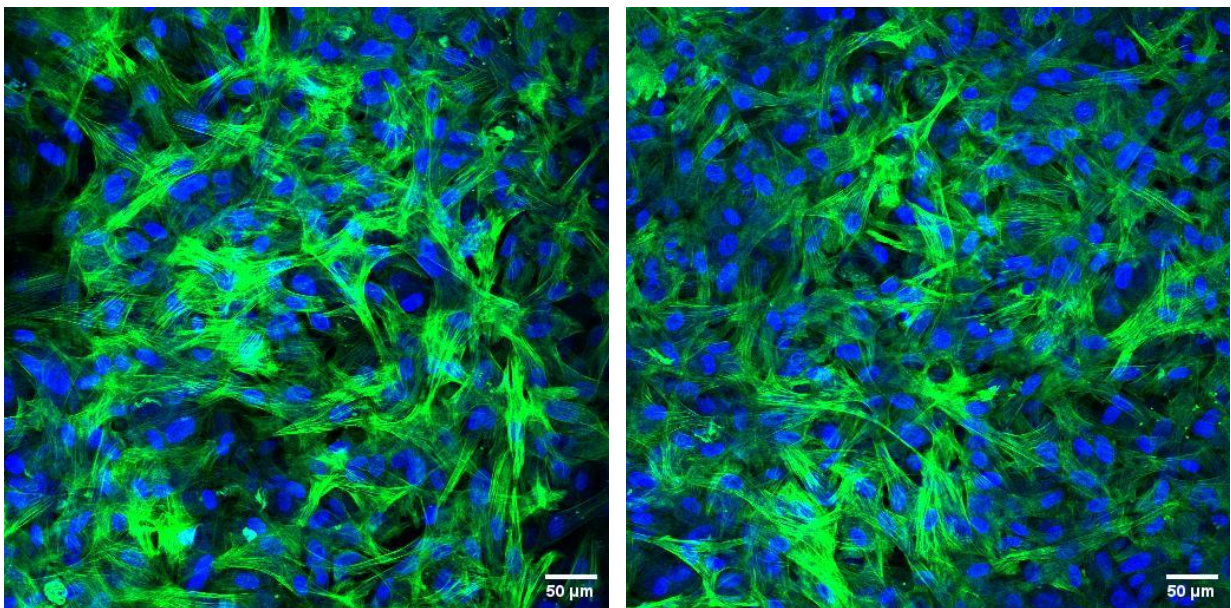


Figure 46: Confocal microscopy images of differentiated HAOSMCs on CellDrums stained with α - smooth muscle actin (green) antibody & Höchst (blue) nuclear stain (20X).

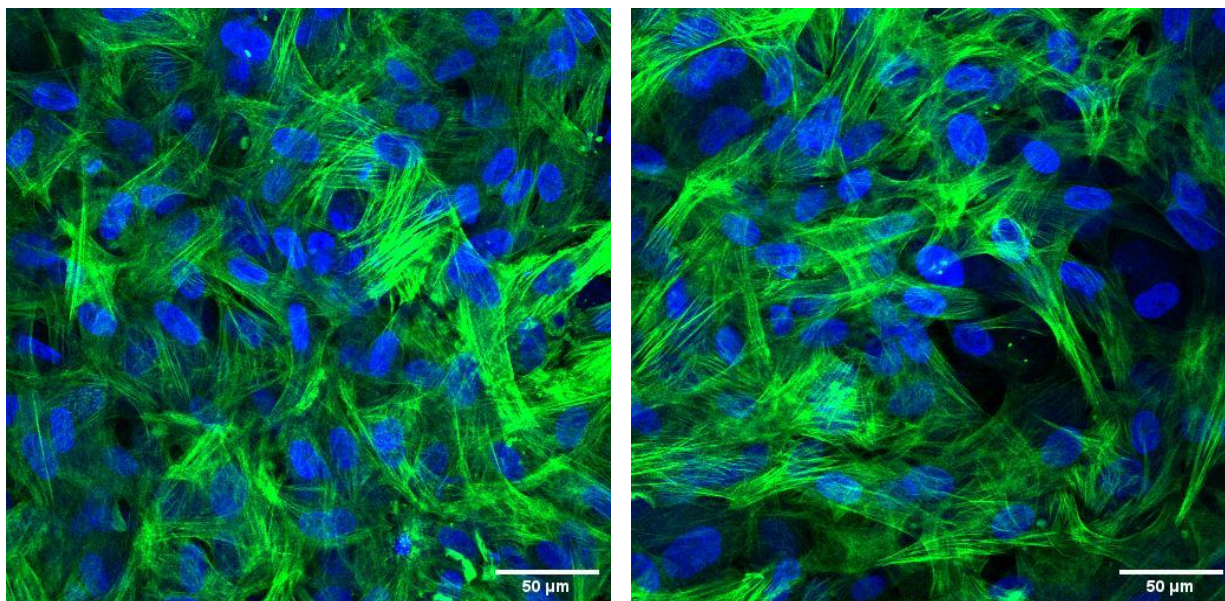


Figure 47: Confocal microscopy images of differentiated HAOSMCs on CellDrums stained with α -smooth muscle actin (green) antibody & Höchst (blue) nuclear stain (40X).

The confocal fluorescence images shown above in Figures 46 and 47 depicts HAOSMCs stained for α -smooth muscle actin (α -SMA, green) and nuclei (Hoechst, blue) at 20X and 40X magnification. The strong fluorescence signal reveals a dense, well-organized filamentous cytoskeletal structure, confirming that the cells exhibit a contractile phenotype. Nuclei are evenly distributed across the monolayer, indicating a healthy and uniform cell population on the CellDrumTM membrane.

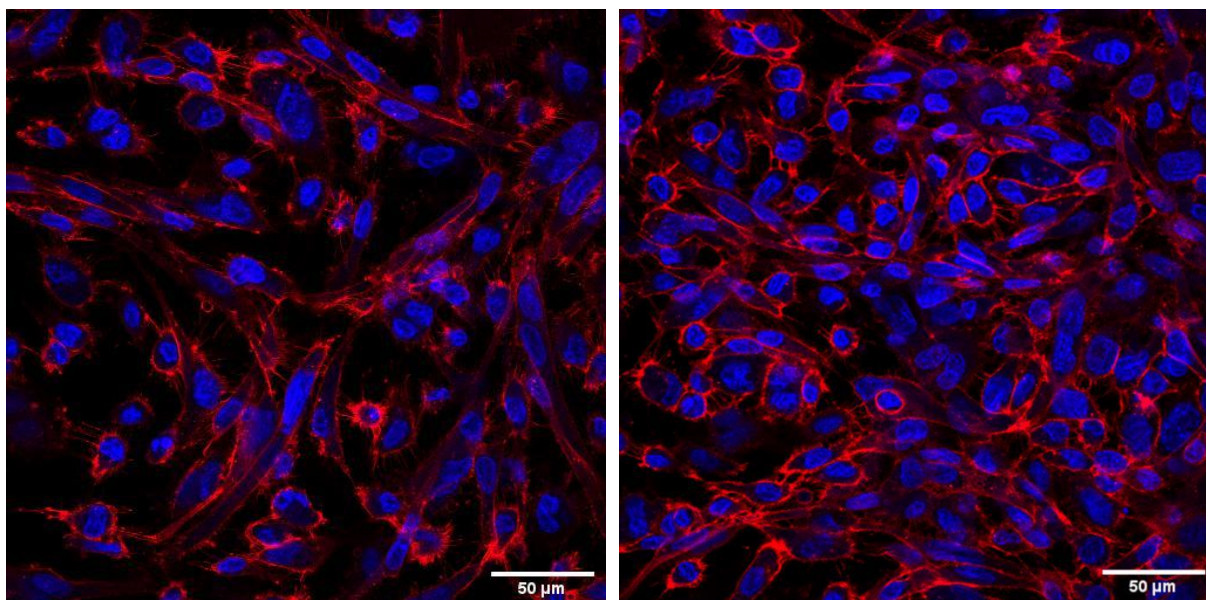


Figure 48: Confocal microscopy images of HAOEC on CellDrums stained with CD31 (red) antibody & Höchst (blue) nuclear stain (20X).

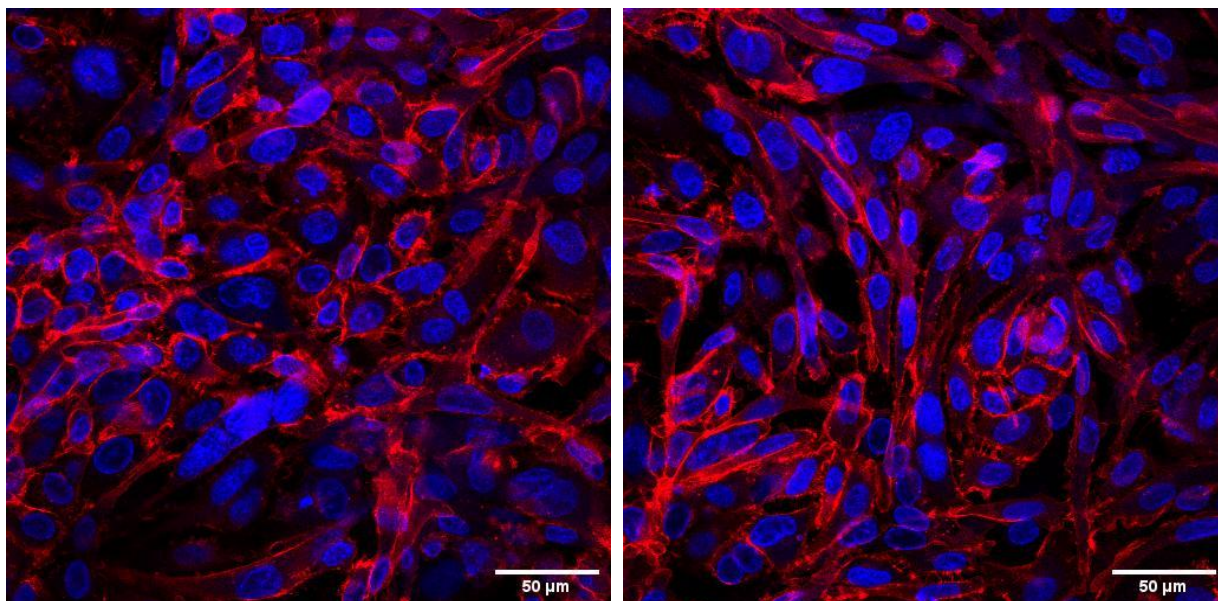


Figure 49: Confocal microscopy images of HAOEC on CellDrums stained with CD31 (red) antibody & Hoechst (blue) nuclear stain (40X).

Figures 48 and 49 display HAOEC monolayers stained with Hoechst and CD31, captured at 20- and 40X magnification using a confocal microscope. CD31 signal can be seen localized at the membranes, consistent with its known role as an endothelial junctional marker. Nuclei are once again well distributed, as with the HAOSMC monolayers presented above.

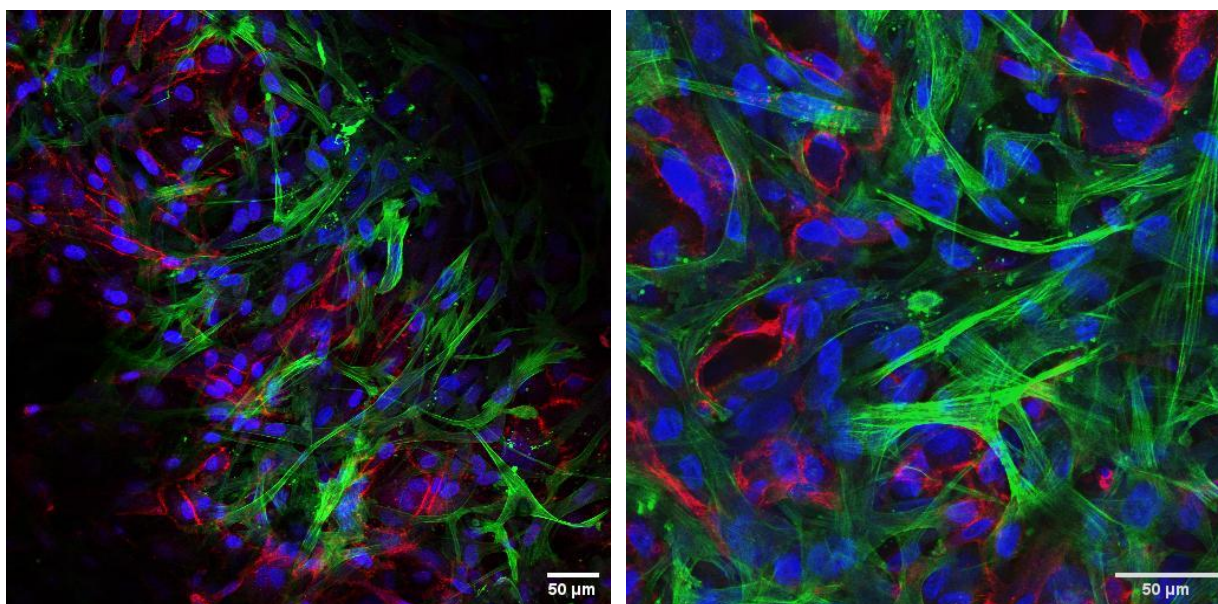


Figure 50: Confocal microscopy images of HAOSMC-HAOEC co-culture on CellDrums stained with CD31 (red), α -smooth muscle actin (green), and Hoechst nuclear stain (blue). Left image: 20X; Right image: 40X.

In the co-culture of HAOSMCs and HAOECs, confocal microscopy images (Figure 50) stained with α -smooth muscle actin, CD31 (red), and Hoechst nuclear dye (blue) exhibit a well-integrated monolayer containing both vascular cell types. CD31 staining is confined to the cell membranes of endothelial cells, which appear as tightly packed, cobblestone-like structures, while α -smooth muscle actin is localized within elongated, spindle-shaped smooth muscle cells,

consistent with their contractile phenotype. The Höchst dye can be seen staining the cell nuclei, showing a well-distributed cell layout.

3.4.5 Contractile Response of HAOSMCs Monolayers

This following section will present the results of contractile response measurements of the HAOSMC monolayers cultured on CellDrum™ membranes and exposed to increasing concentrations of NA and ionomycin. Aim of these experiments was to examine the concentration-dependent mechanical responses of differentiated HAOSMCs, measured by the CFA_{refer} system. Substance induced cellular tensile stress changes were measured to determine dose-response curves and half-maximal effective concentration (EC₅₀) values. All experiments were conducted under controlled and standardized laboratory conditions, including CellDrum™ quality management procedures, cell differentiation protocol, and the measurement setup. The results presented here provide the basis for new insights into the biomechanical responsiveness of isolated HAOSMC monolayers to vasoactive substances and at the same time demonstrate the use of the CellDrum™ platform for investigating vascular cell contractility in-vitro. Since individual CellDrums can have different, visually undetectable pre-tensions despite having the same membrane thickness, an offset correction of these tensions was carried out after the CellDrums had been measured without cells.

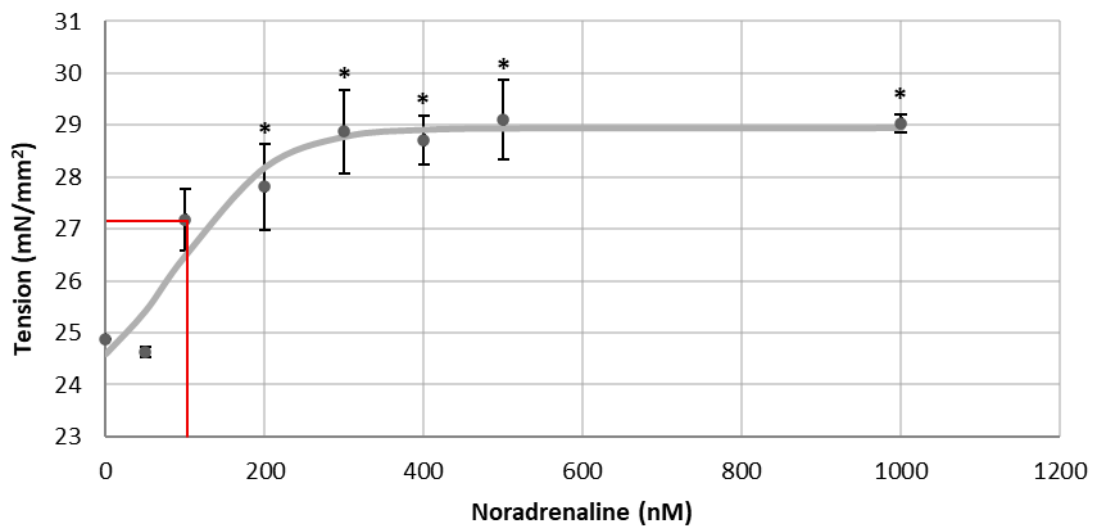


Figure 51: Cellular tensile stress of HAOSMC monolayers vs. Noradrenaline concentration, $n=5$. Friedman Test (non-parametric version of repeated measures ANOVA), * $p < 0.05$, significance compared to control value. The curve depicts sigmoidal fit of the SMC monolayer data. The EC₅₀ calculated from the fit is 100.70 nM (red line).

Figure 51 shows the contractile response of HAOSMC monolayers exposed to increasing concentrations of NA. Treatment with 50 nM NA produced a minor but statistically non-significant decrease in tension relative to control conditions. When exposed to 100 nM NA, a modest tension increase was noted, though this change also lacked statistical significance. The first statistically significant elevation in membrane tension occurred at 200 nM NA. Maximum tension was reached at 500 nM, with saturation evident at higher concentrations. Nonlinear regression analysis of the dose–response curve revealed an EC₅₀ of approximately 100.70 nM and a Hill coefficient of 3.28, indicating steep activation of the contractile response in SMCs.

To verify the contractile capacity of the HAOSMC monolayers and ensure the responsiveness of the measurement system, ionomycin calcium salt was used as positive control. The Ionomycin ionophore elevates intracellular calcium levels by facilitating calcium influx across cellular membranes, thereby inducing contraction of smooth muscle cells independent of receptor-mediated pathways. This mode of action makes it a suitable reference agent for validating the contractile functionality of differentiated HAOSMCs. The same CFA_{refer} measurement protocol described previously for the NA-experiments was applied for Ionomycin measurements to elucidate concentration-dependent mechanical cell responses. All measurements were conducted under identical experimental conditions to maintain consistency and comparability across datasets.

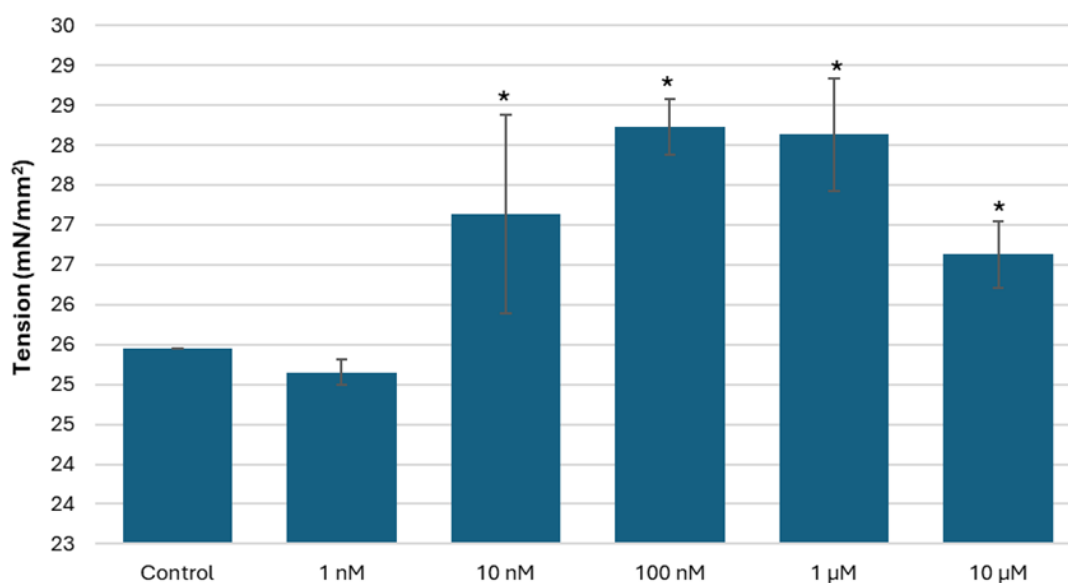


Figure 52: Cellular tensile stress of HAOSMC monolayers vs Ionomycin concentration, $n=4$ (bar chart). Error bars represent SEM values. One-way repeated measures ANOVA with Holm-Sidak correction, $*p < 0.05$, significance compared to control value.

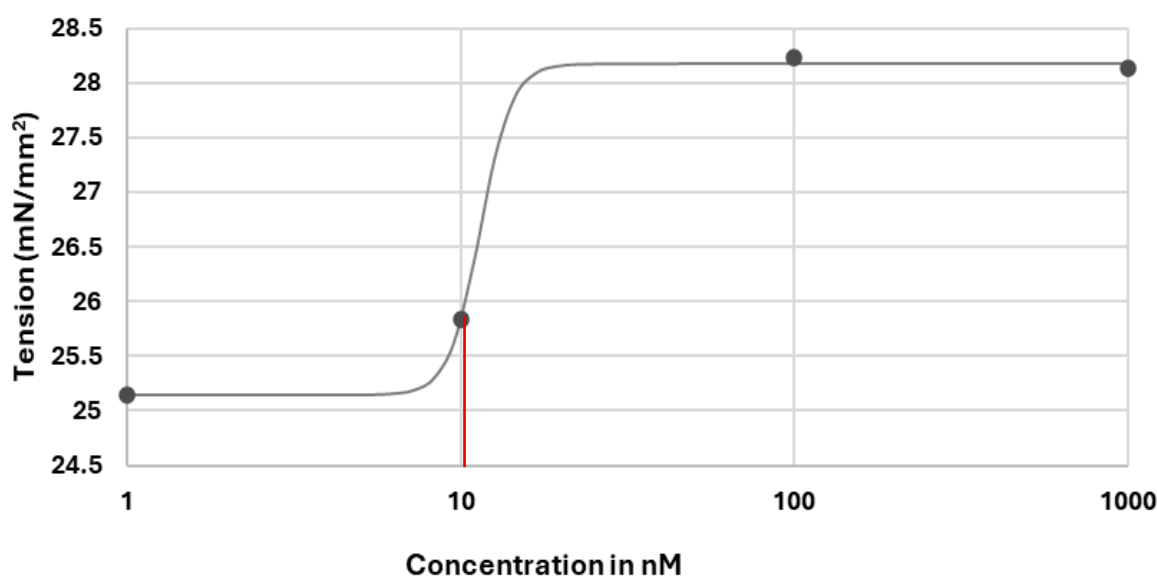


Figure 53: Cellular tensile stress of HAOSMC monolayers vs Ionomycin concentration, $n=4$. The curve depicts sigmoidal fit of the Ionomycin-SMC monolayer data. The EC₅₀ calculated from the fit is 11.44 nM (red line).

The bar chart (Figure 52) provides a comprehensive view of the Ionomycin dose-response relationship with the HAOSMC monolayer. The data shows baseline tension at approximately 25.8 mN/mm² for control conditions, with 1 nM ionomycin producing a slight, non-significant decrease of 1.1% to ~25.2 mN/mm². At 10 nM, a mild increase of 1.5% is observed, while statistical significance markers indicate that concentrations of 10 nM and above (10 nM, 100 nM, 1 μ M, and 10 μ M) all produce significant increases in tension compared to control. A strong increase of approximately 10.9% occurs at 100 nM, indicating a clear dose-responsive effect, which is maintained at 1 μ M with a 10.6% increase, suggesting a plateau near the maximal tension response. At 10 μ M, tension decreases from its maximum yet remains 4.7% above control (~26.8 mN/mm²), indicating a partial reduction that may reflect receptor desensitization.

In Figure 53, the graph shows a nonlinear regression analysis of the ionomycin dose-response data in GraphPad fitted with a four-parameter logistic (4PL) sigmoidal model on a log-transformed x-axis (log [ionomycin concentration]). However, this representation necessarily excludes the control (0 nM) data point due to mathematical constraints of logarithmic transformation, where log (0) is undefined and therefore cannot be accurately positioned on the logarithmic concentration axis. The log-scale clearly demonstrates the EC₅₀ occurring around 11.4 nM (marked by the red line at the curve's inflection point) and shows the plateau saturation at approximately 28.1 mN/mm² above 100 nM, making it particularly valuable for pharmacological analysis despite the limitation of excluding zero and very low concentration values.

3.4.6 Contractile Response of HAOSMC-HAOEC Co-cultures

This section focuses on the contractile response of HAOSMC-HAOEC co-cultures exposed to NA and Ionomycin. The question here was to what extent the addition of an endothelial cell layer to a smooth muscle cell monolayer would alter the cellular mechanical tension under otherwise identical circumstances. Using the same standardized CFA_{refer} measurement protocol as described previously, including membrane selection, differentiation regimen, and substance exposure sequence, the co-cultures were tested for their dose-dependent changes in membrane tension. By employing this co-culture setup, the aim was to better mimic the cellular architecture of vascular wall tissue and explore potential modulatory effects of endothelial cells on smooth muscle contractility.

Offset correction was similarly applied for graphical normalization. However, unlike the HAOSMC monolayer results, the co-culture data did not conform to a classic sigmoidal dose-response relationship. Despite the attempts to fit the co-culture contractility data using various nonlinear regression models like biphasic and asymmetric five-parameter logistic (5PL) fits, none yielded an adequate coefficient of determination (R²). The asymmetric model, while showing a modest improvement over others, resulted in an R² of approximately 0.70, which was considered insufficient for reliable curve modelling and accurate parameter estimation. Consequently, no curve fit was applied to this dataset.

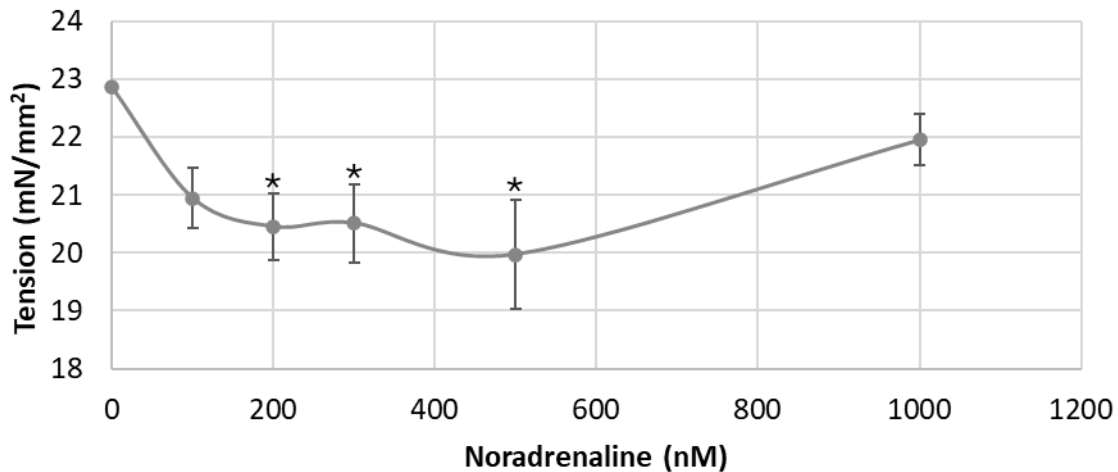


Figure 54: Cellular tensile stress of HAOSMC-HAOEC **co-cultures** vs. Noradrenaline concentration, $n=3$. Error bars represent SEM values. Friedman test with Dunn's correction: * $p < 0.05$, significance compared to control value.

Figure 54 shows that NA induced a concentration-dependent, biphasic effect on membrane tension in HAOSMC–HAOEC co-cultures ($n = 3$). At baseline (control), the co-cultures exhibited the highest measured tension (~ 23 mN/mm²). Treatment with 50–500 nM NA resulted in a significant reduction in tension ($p < 0.05$), reaching a minimum at 500 nM (~ 20 mN/mm²). At 1000 nM, tension increased again, approaching values closer to baseline. This pattern suggests that low-to-moderate concentrations of NA reduce cellular contractility, while higher concentrations partially restore tension. Due to the biphasic nature of the dose-response curve, a clear EC₅₀ value could not be determined.

Ionomycin was expected to provide a robust stimulus triggering contraction of the co-culture due to its receptor-independent mechanism of action. The same experimental procedures used in the HAOSMC monolayer assays were applied here, ensuring consistency and enabling direct comparison between mono- and co-culture responses under controlled conditions.

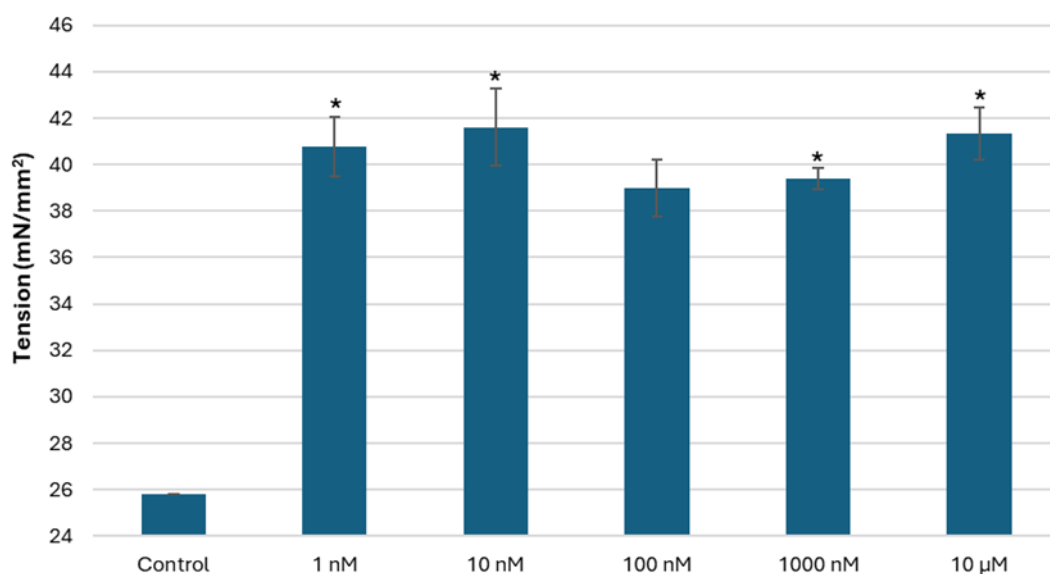


Figure 55: Cellular tensile stress of HAOSMC-HAOEC **co-cultures** vs Ionomycin concentration, $n=2$ (bar chart). Error bars represent SEM values. Friedmans test with Dunn's correction: * $p < 0.01$, significance compared to control value.

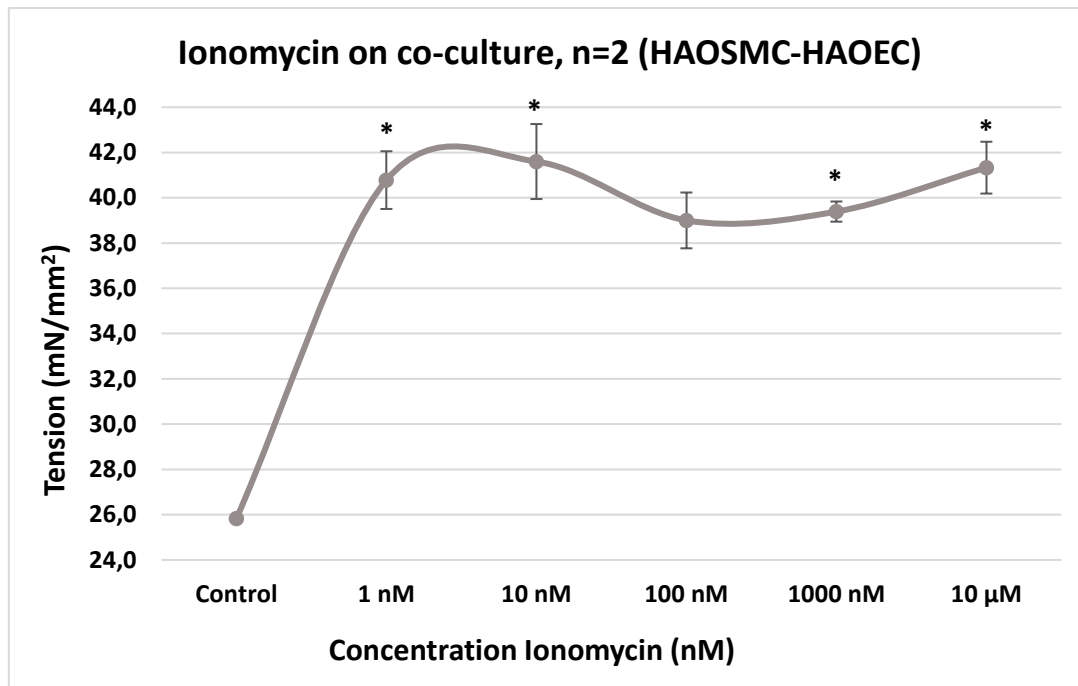


Figure 56: Ionomycin effects on the tensile tension of HAOSMC-HAOEC *co-cultures*. Error bars represent SEM values. Friedman test with Dunn's correction: *p*-values control vs 1nm (0.0042), vs 10 nM (0.0002), vs 1000 nM (0.0164), 10 µM (0.0005).

Figures 55 and 56 display the dose response effects of Ionomycin on the tensile stress of HAOSMC-HAOEC co-cultures. At 1 nM, tension increases by 58%, indicating a strong sensitization at very low ionomycin concentrations. This further increases to a 61% rise at 10 nM, representing the maximum observed response in this experiment. At 100 nM, tension remains elevated with a 51% increase relative to control, showing a slight decrease from the peak while maintaining high contractility. At 1000 nM, tension increases by 53%, indicating a stable plateau at higher doses. At 10 µM, tension rises again to a 60% increase relative to control, returning to near-maximum levels seen at lower concentrations. A sigmoidal fit was not applicable as the response rapidly saturated at low concentrations without a gradual dose-dependent transition. The system achieved maximal activation at the lowest tested concentration (<1 nM), indicating an EC50 below this value.

4. Discussion

This section will address the results referenced earlier, divided into the three phases. Although some trends were consistent across replicates, there were also deviations that may indicate either biological variability or technical limitations inherent to the system. It is important to acknowledge that although effort was made to standardize procedures of CellDrum™ production, some degree of variation between CellDrum batches was difficult to completely eliminate.

4.1 Phase 1- CellDrum™ Production and Optimization

In the first phase of this study, the objective was to optimize not only the CellDrum™ membrane but the entire CellDrum™ system including the applicators. As part of this effort, a material screening process was carried out to explore alternatives for the CellDrum™ applicators. The goal was to improve the adhesion properties of the PDMS membrane and also to identify a more cost-effective material. This was especially relevant given that the currently used PEEK material is relatively expensive, which could become a limiting factor if commercial production of CellDrums is considered in the future. The selected material was subjected to SEM imaging as well as an in-vitro biocompatibility assay, and the results presented in sections 3.1.1 and 3.1.2 will be discussed below.

A clear difference in surface appearance was observed between the PP-HOMO and PEEK CellDrum™ applicators under SEM analysis. The circular fiber structure was more distinctly visible in the PP-HOMO samples across all magnifications and signal modes, which can be due to differences in production methods: while the PEEK applicators were produced by machining, the PP-HOMO applicators were manufactured using injection moulding. The surface of the PP-HOMO material appeared smoother overall compared to the PEEK applicators, likely due to the combination of the production method and the fact that the PP-HOMO applicators were only a few months old and had undergone minimal use, whereas the PEEK applicators had been in frequent use for approximately three years, leading to visible wear and tear.

Minor surface impurities and heterogeneities were also observed on the PP-HOMO applicators at higher magnification, but these were significantly less pronounced than on the older PEEK surfaces. Importantly, such small surface irregularities are unlikely to affect the quality of the CellDrum™ membranes or the membranes' ability to adhere to the applicator surface. This assumption is further supported by the fact that the older PEEK applicators, despite displaying more pronounced surface heterogeneity, have not shown any issues with membrane attachment or stability during experimental use to date. Overall, the PP-HOMO applicators demonstrated a surface quality suitable for CellDrum™ applications while offering the potential for cost-effective and scalable production.

The SEM examinations performed by the GFE provided valuable insights into the surface characteristics of both PEEK and PP-HOMO CellDrum™ applicators, revealing differences owed to production methods, material properties, and usage-dependent wear. However, to further improve the quality assurance of future CellDrum™ applicator production, acquiring 3D topographical data would be beneficial. Unlike 2D SEM images, 3D imaging can provide quantitative measurements of surface features, including their height, offering a more detailed assessment of surface impurities. When combined with specific filters, 3D topographic images

could be generated, providing quantitative measurements of surface structures beyond what is possible with 2D SEM imaging. Should quantitative data not be feasible via filtering, more detailed analyses using atomic force microscopy (AFM) or profilometry could be performed to obtain high-resolution surface information. Although this study does not directly address the PEEK and PP-HOMO materials and their use with PDMS, Borók et al. (2021) emphasize the critical role of surface properties and treatment in ensuring the functional quality of PDMS-based microdevices, supporting the relevance of systematically evaluating surface characteristics when developing and optimizing components such as the CellDrum™ system for reliable membrane attachment and reproducible performance ¹⁴².

The choice of surface examination technique should be guided by the specific quality parameters deemed critical for CellDrum™ applicators. AFM provides high-resolution roughness data but is limited to small sample areas, while stylus profilometry offers larger scan areas at the expense of missing fine surface details due to tip size ¹⁴³. Recent findings confirm that while profilometry allows faster scanning over larger areas, AFM offers higher-resolution surface characterization, particularly valuable for detecting fine topographical features, with both methods providing complementary data essential for the quality assessment of biomaterial surfaces (Mei et al., 2023) ¹⁴⁴. It is also essential to evaluate how these measured parameters correlate with practical aspects such as membrane attachment quality, applicator durability, and production scalability, especially considering the high operational costs and potential delays these measurement systems could introduce if applied to every unit during production.

Furthermore the demonstrated biocompatibility of PEEK in combination with PDMS in biomedical applications (Smith et al., 2019) further supports its current and future use in the CellDrum™ system, where mechanical stability and biocompatibility in combination with cell cultures are critical for in-vitro wound healing as well as mechanobiology studies ¹⁴⁵.

The biocompatibility tests demonstrated consistently high cell viability across both the control and experimental groups throughout the four-day cultivation period. As shown in the bar chart in Figure 25, most conditions exhibited cell vitality rates above 95%. By day 4, all groups, including 4M, exhibit similarly high vitality, indicating recovery or adaptation over time. The absence of significant differences in cell viability across groups underscores the high biocompatibility of PP-HOMO. Even prolonged exposure at higher concentrations did not impair cell vitality, highlighting the material's suitability for long-term cell culture applications and supporting its potential use in tissue engineering contexts.

Overall, the results seen in section 3.1.2 show that the presence of the PP-HOMO material within the culture environment did not negatively impact cell viability or proliferation. On the contrary, the vitality rates in the experimental groups were marginally higher than in the controls, suggesting that the material does not release cytotoxic components and is well-tolerated by the cultured human dermal fibroblasts. Additionally, a collaborating research group (RG Saric, Uniklinikum Köln), working with cardiomyocytes tested the same PP-HOMO material and similarly reported no negative effects on cell health, further supporting the biocompatibility of the material. These findings actually confirm the suitability of the PP-HOMO material for use in the CellDrum™ system without compromising cellular health or experimental reliability during in vitro applications.

4.2 Membrane Production and Optimization

Following the initial optimization of the CellDrum™ applicators, subsequent experiments focused on refining the membrane fabrication process. In this part of the study, the dependency of membrane thickness on key fabrication and mechanical factors was examined. Specifically, the effects of PDMS volume, curing temperature, membrane deflection and compliance were analysed to better understand how these parameters influence membrane characteristics relevant to CellDrum™ performance.

4.2.1 Dependency of Membrane Thickness on PDMS Solution Volume

Three different volumes of PDMS solution were tested for this experiment: **4, 8, and 10 μ L**. Volume adjustment represents the most direct and practical method for controlling membrane thickness, and these proportional volumes were selected to analyse correlations between the resulting groups. Based on the mathematical formula for calculating membrane thickness previously employed by Kim et al.¹⁹, a linear increase in thickness values was expected with increasing PDMS volume. However, the measured thicknesses did not fully align with the theoretical predictions, as shown in Table 6.

These deviations from theoretical values can be due to several factors. One possibility is that the PDMS droplet does not spread in a perfectly circular and uniform manner and/or potentially resulting in thicker regions toward the center. This may be further affected by non-uniform curing due to uneven heat distribution from the curing lamps, which can lead to localized variations in membrane polymerization. To address this issue, alternative curing methods such as oven curing could be considered, where the entire chamber or room is heated to the required curing temperature, ensuring more uniform heat distribution throughout the membrane surface. However, since these membranes are cured in plastic multi-well dishes, such increase in environment temperature must be carefully adjusted.

Imprecise positioning of the floating PDMS drop away from the center could also contribute to uneven elastomer distribution. This positioning issue could be addressed through the implementation of mechanical positioning systems, such as XY-stages with precise micrometers, or guided pipetting fixtures that maintain consistent positioning relative to the dish center. Visual alignment aids, such alignment marks or liquid dyes, could also improve drop placement accuracy. Additionally, environmental disturbances such as air currents over the water surface may affect spreading uniformity, suggesting that the membrane curing process should be isolated from room ventilation systems. The hypothesis of non-uniform PDMS spreading was supported by thickness measurements taken at six circular points using the specialized holder (Figure 19), which revealed variations across membrane surfaces. However, these variations remained below 10%, maintaining thickness distribution within a 90% confidence interval, and therefore are not discussed in further detail in this study.

Figure 26 shows a strong positive correlation between PDMS volume and membrane thickness in the 4-10 μ L range ($r^2 = 0.97$, $p < 0.01$), with significant differences between all groups. In contrast, no such relationship was observed in the narrower 3-4 μ L range. These results indicate that membrane thickness can be reliably tuned by adjusting PDMS volume, but only across larger volume ranges.

4.2.2 Dependency of Membrane Thickness on Room Temperature

To assess the influence of ambient conditions on membrane formation, PDMS membranes were produced using a constant volume of 4.15 μL while setting the room temperature to 22, 24 and 26°C respectively, representing typical seasonal laboratory conditions. Temperature was carefully regulated during each trial to ensure consistency throughout the fabrication process.

The viscosity of liquids, including those spreading on water surfaces, exhibits an inverse relationship with ambient temperature, which accounts for the observed increased central thickness in membranes fabricated at lower temperature. This temperature-dependent viscosity effect directly influences the spreading behaviour of PDMS, with visibly slower spreading rates observed at lower temperatures compared to higher temperatures^{141,146}. The reduced fluidity at lower temperatures limits the elastomer's ability to achieve uniform distribution across the water surface, resulting in thicker central regions. Additionally, temperature not only affects viscosity but also influences the surface tension of PDMS as well as the underlying water volume, both of which decrease with increasing temperature¹⁴⁷. This dual reduction facilitates improved spreading of PDMS films, particularly in the temperature range relevant to laboratory environments. Experimental data (Darhuber et. al, 2003) from previous studies show that even small changes in temperature, especially below 40 °C, can actually cause disproportionately large variations in both viscosity and surface tension, further supporting the sensitivity of membrane uniformity to ambient conditions^{146,147}.

Despite the relatively narrow temperature range examined in this study, statistically significant differences in membrane thickness were observed. Between groups 1 and 3, with only a 4 °C ambient temperature difference, membrane thickness decreased significantly (One-way ANOVA, $\alpha = 0.01$) by approximately 10% (Table 7, Figure 27). This substantial change over such a small temperature range demonstrates the critical sensitivity of the fabrication process to environmental conditions, highlighting the importance of temperature control during membrane production to ensure reproducible results. Therefore, PDMS should be dispensed at constant ambient temperature within a controlled environment, such as a closed container or climate-controlled chamber. This approach maintains consistent temperature conditions while providing additional benefits like dust-free air, reduced air currents and improved fabrication reproducibility. Temperature monitoring and control systems during production would also enable quality assurance and batch-to-batch consistency in membrane characteristics.

4.2.3 Membrane Functionalization

The successful adhesion of the cultured cells to the surface of the CellDrum membrane is an essential prerequisite for the precise functioning of this technology for measuring cell force. The effectiveness of the surface modification methods applied to CellDrumTM membranes has been demonstrated through previously conducted surface tension analyses, which evaluated critical surface energy (γ_c , in mJ/m^2) as a measure of wettability¹⁸. In the study by Trzewik et al., membranes without surface treatment were compared to those subjected to plasma etching and subsequent fibronectin coating. The findings confirmed that the functionalization process significantly increased membrane wettability, supporting successful cell adhesion¹⁸. This improvement is attributed to both the enhanced surface energy resulting from plasma treatment and the cell-adhesive properties of fibronectin, a commonly used ECM protein.

While no universal definition of biocompatibility exists, within the scope of this thesis the term refers to the membrane's capacity to support stable cell attachment, survival, and proliferation under standard and experimental conditions. The chemically and biologically functionalized PDMS surfaces used in the CellDrum™ system have repeatedly demonstrated this capability across various studies. Cells not only remained viable over prolonged cultivation periods but also responded appropriately to mechanical stimulation and pharmacological treatments^{19,20,138,148,149}. Moreover, the membrane has proven compatible with a broad spectrum of cell types, including vascular smooth muscle cells (from coronary, pulmonary, and arterial origins), endothelial cells (from both coronary and arterial sources), dermal and 3T3 fibroblasts, human and rat cardiomyocytes, and even complex vascular co-cultures^{18,19,24,25,29,130}.

In addition to the published data, numerous internal laboratory studies have consistently demonstrated the reliability of the membrane functionalization protocol^{19,26,29,47}. Across a variety of experimental setups and cell types, no issues were encountered regarding cell attachment, viability, or proliferation on the modified membrane surfaces. These cumulative findings further validate the chemical and biological coating procedures employed in CellDrum™ preparation, underscoring their robustness and reproducibility in supporting long-term cell culture applications.

4.2.4 Interdependency of Membrane Thickness and Deflection

The relationship between membrane thickness and mechanical behaviour was explored through a two phased experimental approach. First, passive membrane deflection under static conditions was analysed to assess how thickness influences the mechanical response of the membrane. In the second part of the experiments, the link between membrane thickness and membrane compliance was further investigated using the CFA_{refer} measurement system. These experiments provided a baseline understanding of how physical properties such as membrane stiffness are affected by dimensional changes.

As shown Figure 29, membrane deflection was measured across five thickness groups ranging from ~3.28 to 3.98 μm . Although minor fluctuations in average deflection values were observed, no statistically significant differences were detected between groups. This suggests that within this narrow thickness range, membrane tensile stress, as reflected by membrane deflection, remained relatively stable. Figure 30 shows a different pattern compared to what was seen in the previous figure, demonstrating a clear inverse relationship between membrane thickness and membrane deflection. Membranes with an average thickness value of 3.47 μm exhibited significantly higher deflection compared to those with thicknesses of 7.49 μm and 9.43 μm ($p < 0.01$) as expected. No significant difference was observed between the 7.49 μm and 9.43 μm groups, suggesting a plateau in deflection reduction beyond a certain thickness at the hydrostatic load chosen. These findings indicate that increases in PDMS membrane thickness in larger thickness ranges may have a diminishing effect on reducing membrane compliance.

In 2009, Liu et al. investigated the thickness dependency of PDMS membranes' mechanical properties, produced by spin coating method. These characteristics were previously considered to be dimensionally independent¹⁵⁰. His research group revealed as expected that the Young's modulus as well as the general mechanical strength of the PDMS membranes to be thickness dependent. They defined ranges for different mechanical behaviours known as bulk behaviour

and dimension dependent behaviour ($<200\text{ }\mu\text{m}$). This transition in behaviour is found to be correlating to a thickness dependent reorganization of the polymer chains to create more resilient cross-linked networks, during membrane production. All membranes used in our study were significantly below the transition value of $200\text{ }\mu\text{m}$ which fit the thickness dependent behaviour range. However, there was no linearity or pattern found between the two investigated parameters in the thickness range of $3.28\text{--}3.98\text{ }\mu\text{m}$. The use of such small thickness range with might be the reason why no significant results were obtained for the first deflection trial as seen in Figure 29.

The thinnest membrane used in the study by Liu was $30\text{ }\mu\text{m}$, while ours is roughly about 1/10th of that value. That could be the initial reason to the lack of correlation between membrane thickness and deflection values. Despite that, the second deflection trial (Figure 30) with the bigger thickness range ($3.24\text{--}10.06\text{ }\mu\text{m}$) showed significant dependency of measured deflection values and the membrane thickness. The results prove that wider ranges of membrane thickness must be chosen for investigation for ultra-thin membranes, supporting the hypothesis of Liu et al. on dimension dependent behaviour.

Another study from 2014 by Johnston et al. studied the dependency of membrane mechanical properties such as Young's modulus, ultimate tensile strength (UTS), ultimate compressive strength (UCS) and compressive modulus on curing temperature¹⁵¹. He reported significant differences for these parameters in a temperature range of $25\text{--}200\text{ }^{\circ}\text{C}$. In the study, $200\text{ }\mu\text{m}$ thick membranes were used. Membranes above $200\text{ }\mu\text{m}$ thickness are believed to show bulk behaviour. However, Johnston et al. revealed an increase in the Young's Modulus (MPa) of the PDMS membrane with increasing curing temperature. Membrane mechanical properties were also proven to be dependent on curing temperature. Therefore, it is highly likely that these properties could also be influenced by ambient room temperature as reported earlier (Figure 27, section 3.2.2). Even though ambient room temperature was kept as stable as possible for the production of all membranes for the deflection measurements, slight fluctuations ($\pm 0.5\text{ }^{\circ}\text{C}$) could have occurred. These fluctuations could have had an additional effect on the membrane tensile stress and therefore the deflection values.

Moreover, to once again display how the CellDrum membranes can differ in mechanical characteristics under identical production parameters, an additional calculation was carried out. Five membranes with the same thicknesses (differing only in 3rd decimal) from the first deflection trial ($n = 35$) were chosen and their measured deflection values were examined. These membranes had an average thickness of $3.648 \pm 0.004\text{ }\mu\text{m}$. Deflection values for each membrane were as follows; $916\text{ }\mu\text{m}$ (1), $1378\text{ }\mu\text{m}$ (2), $939\text{ }\mu\text{m}$ (3), $569\text{ }\mu\text{m}$ (4) and $1008\text{ }\mu\text{m}$ (5), respectively. These results prove once again that the membrane thickness is not the only parameter which affects mechanical membrane properties. As mentioned before, a pretension is provided to the membrane as it is stretched over the CellDrum applicator. This pretension is a determinant factor of membrane deflection as it directly influences how the membrane reacts to external additional loads on top of the surface. The membrane transfer from the mechanical aid onto the applicators is a manual procedure. Therefore, deviations in the intensity of this pretension are expected. An automated procedure can remedy this situation. Preparations for this are currently underway⁴⁷. The automatization is expected to decrease or completely prevent unequal distribution of forces during the mechanical transfer of the membrane, minimizing possible inconsistencies.

4.2.5 Interdependency of Membrane Thickness and Compliance

As described in Section 4.2.4, the relationship between membrane thickness and mechanical properties was examined under static and dynamic conditions. Visual inspection suggested little interdependence between thickness and compliance below 4 μm , likely due to other dominating factors at such low thicknesses. As shown in Figure 31, membranes across the 3.20-3.97 μm range exhibited only minor, non-significant differences in compliance, indicating relative mechanical stability within this interval. Liu et al. found in their experiments that membrane thickness values below a threshold of 200 μm tend to shift from bulk behaviour to dimension dependent behaviour. This might be due to a thickness dependent reorganization of the PDMS polymer coils¹⁵⁰. However, their tests were performed at much coarser thickness intervals and never came close to our membrane thicknesses. Rather, production circumstances such as curing temperature might have a considerable large influence on the mechanical properties, as pointed out by Johnston et al.¹⁵¹. These factors might even shadow the influence of thickness on these membranes.

Ultimately, the current understanding of the mechanical and structural properties of ultra-thin PDMS membranes remains limited, underscoring the necessity for rigorous quality control measures and continued systematic investigation to ensure consistency and reliability in their application.

4.2.6 Membrane Stiffness Comparison to In Vivo

The compliance of 30 measured CellDrumTM membranes obtained by the CFA_{refer} device averaged a value of $5.3 \times 10^6 \text{ Pa}^{-1}$, which equates to a stiffness value of approximately 190 kPa. In comparison, tests of the stiffness of PDMS of the same blend claimed to have shown values of 1.32–2.97 MPa¹⁵². This indicates that the here used production methods might have lowered the materials stiffness substantially. Nonetheless, the results should be taken with a grain of salt, since there seems to be a rather large variation of stiffness of PDMS depending on the production method. As the CellDrumTM membranes are also meant to be used as culture vessels, these should be compared to commonly used tissue culture plates/flasks made of Polystyrene, and to tissue types found in the human body. Skeletal muscle and cartilage have an stiffness of 12 and 42 kPa respectively^{153,154}. Thus, PDMS membranes are still considerably stiffer than these tissues. However, compared to polystyrene bottles with a stiffness of 10 MPa, the PDMS membrane is still preferable. Furthermore, the PDMS membranes showed to be easily functionalized with protein coatings. If low surface stiffness is demanded, coating the membrane with a low stiffness hydrogel, for instance, could be easily fulfilled.

Despite the high precision production technique of the CellDrums, it is especially challenging to select a six-piece batch of membranes whose base compliance do not exceed 3% deviation. For this purpose, the individual CellDrums were numbered with a code that include mechanical parameters. The parameters (thickness, compliance) are used in the final individual CellDrumTM product sheet in order to calculate the mechanical stress of cell and tissue layers. This is the only way to ensure comparable and repeatable measurement of lateral cell forces of cultured cell monolayers.

4.3 Phase 2- Wound Healing Assay

4.3.1 CellDrum™ Membrane Production

Consistency in the preparation of CellDrum™ membranes is important to ensure reliable experimental outcomes. To achieve this, a standardized laboratory procedure including visual inspection, quality control, and thickness measurements was followed (section 2.3). Approximately 84% of all CellDrums manufactured in this way were usable for further experiments.

The membrane thickness across all experimental groups remained highly consistent as seen in Table 11, with average values ranging narrowly between 3.66 and 3.70 μm . The low standard error of the mean ($\text{SEM} \leq 0.10 \mu\text{m}$) across all groups indicates excellent reproducibility in membrane production. The box plot in Figure 38 confirms that membrane thickness was maintained within a narrow and reproducible range across all study groups, regardless of donor age or experimental condition. The similarity in median and average values, as well as the overlapping interquartile ranges, indicate that membrane fabrication was uniform and unaffected by grouping factors. This consistency ensures that any observed differences in cellular behaviour can be confidently attributed to experimental conditions rather than variability in membrane thickness and confirms that membrane thickness findings from our laboratory was effectively controlled and remained uniform across conditions.

4.3.2 Cell-Free Gap Perimeter Standardization

In order to determine the uniformity of the created wound area (cell-free gap) by the utilized steel ball technique, the wound perimeter was chosen as the investigated parameter.

While the majority of wounds fell within an acceptable and comparable range, two outliers, one in the 28-year-old control group and one in the 28-year-old training group were excluded due to abnormally large perimeters exceeding 1100 μm . These deviations likely resulted from unintended displacement of the steel ball used during wound creation, particularly during sample transport. This phenomenon is even more likely when samples are handled on rigid substrates, such as plastic flasks as opposed to the more stable, flexible surface of the CellDrum™ membrane⁶. The discrepancies between the highest and the lowest values could also be attributed to the surface tension created by the cell monolayer on the PDMS surface being not always uniform as primary fibroblast cells from different donors tend to have varying capacity to form clusters which are heightened with serum deprivation¹⁵⁵.

Excluding these outliers, the observed wound perimeter ranged from approximately 410 μm to just over 1000 μm , with mean perimeters across all groups remaining closely aligned between 620 and 750 μm . Importantly, no significant differences were found between groups derived from younger (28-year-old) and older (88-year-old) donors. This suggests that the wound induction procedure was sufficiently standardized and did not introduce systematic bias related to age or treatment group. Thus, any differences observed in wound closure dynamics can be interpreted independently of initial wound size variation.

4.3.3 Cell-Free Gap Perimeter Change as Function of Wound Closure

Wound closure dynamics were assessed by measuring changes in the perimeter of the cell-free gap over time, using fluorescence microscopy. The gap created by the steel ball was manually traced, and perimeter values were obtained through the microscope's software across six defined time points (see section 2.6). This approach was consistently applied to all ten samples per experimental group.

Fluorescence images captured on day 4 appear noticeably less defined compared to earlier time points. This reduced clarity is attributed to the declining stability and intensity of the CellTracker dye, as its fluorescence signal diminishes over time. Such signal degradation is a known limitation of cell-permeant dyes and is consistent with the expected photostability profile, particularly beyond 72 hours post-labelling.

The time-dependent wound perimeter data reveal distinct differences in gap closure dynamics between age groups and treatment conditions (Figure 34 and 35). In 28-year-old fibroblast cultures, the control group showed a progressive increase in wound perimeter over time, suggesting limited closure and potential peripheral cell retraction. In contrast, the mechanically stimulated (training) group demonstrated an initial reduction in perimeter by 24 hours, followed by a notable 33% reduction by day four, indicating enhanced wound closure.

Conversely, the 88-year-old donor fibroblasts exhibited more divergent behaviours. The control group showed relatively stable perimeter values in the early time points, with a pronounced reduction at day four, achieving a 55% closure rate, which was the highest among all groups. Interestingly, mechanical stimulation in the older group led to the opposite effect, with the wound area expanding over time, ultimately increasing by 49% at day four. This suggests that mechanical loading using our PulSElect mechanical stimulation device may impair rather than promote wound healing in aged fibroblasts under the conditions tested ²⁴.

For statistical analysis, the one-way ANOVA was performed. It yielded a p-value of 0.0007, indicating a statistically significant overall difference among the groups. The post-hoc analysis revealed that the most significant differences occurred between the trained 28-year-old group and the trained 88-year-old group ($p = 0.001$), as well as between the trained and control groups within the 88-year-old donor set ($p = 0.002$), both of which were highly significant at the $p < 0.01$ level. All other pairwise comparisons were found to be statistically insignificant ($p > 0.05$). These findings suggest that the effects of mechanical stimulation on wound closure dynamics could be age-dependent, with older fibroblasts responding in a fundamentally different manner compared to younger cells. Furthermore, while younger fibroblasts may benefit from moderate mechanical stimulation, aged fibroblasts may respond less favourably, or even negatively, to the same stimulus. These age-related differences in mechanosensitivity should be considered when designing regenerative strategies involving mechanical cues.

As aforementioned, crucial functions of the skin are determined by the mechanical properties of the dermal tissue, which regulates the skin's elasticity and opposition to stretch. Dermal fibroblasts and their activities are profoundly affected by the surrounding ECM structure. Consequently, ECM stiffness governs cell differentiation, proliferation, gene expression, and migration, and thus stability and appearance of the skin/tissue ⁸¹. Mechanotransduction of mechanical cues is fundamental for maintaining cell shape, directing migration, ECM

organization, adapting cell morphology, and many more ^{156,157}. Therefore, biomechanical cues resulting from cyclic stretch used in this study cause both cell-cell and cell-ECM interactions. Dermal fibroblasts adapt and handle this stressful condition by rearranging F-actin structures together with collagen fibers parallel to the load axis forming stress fibers ⁸¹.

The external mechanical stimulation used in this study could have mimicked a wound contraction environment which naturally occurs during the in vivo wound healing process. This stimulatory effect could further explain the positive gap closure rate of the younger donor group. When a skin wound heals, fibroblasts produce stress fibers enhancing tensile forces which allow the closing of a wound and a rapid total blockage of the wound with the help of the secreted collagen and other ECM components ⁷¹.

In our previous unpublished study, the gene expression of type I collagen (COL1A1) was upregulated by a fold change of 2.6 in 28-year-old donor fibroblast cells after mechanical stimulation while the 88-year-old donor fibroblasts showed a reduction of COL1A1 by 66%. The same cell lines (28, 88-year-old donors) and mechanical stimulation parameters/system were used in this study. The mechanical stimulation used in this study could have simulated a late-phase injury environment in 28-year-old donor fibroblasts, which could have resulted in the increased fibroblast proliferation followed by higher collagen expression leading to better ECM remodelling, thus bigger and more significant wound closure in comparison to the control group ^{28,82,90,94}. Many past studies confirm the hypothesis of an increase in COL1A1 after exposure to uniaxial mechanical stretching in cardiac and ligament fibroblasts ^{82,90,94}. More recent and reliable studies using similar cyclic mechanical stresses showed accelerated proliferation and migration of dermal fibroblasts, adipose-derived stromal cells, mesenchymal stem cells, and chondrocytes in comparison to rigid cell culture ^{27,28,158,159}.

An unexpected observation was made in the experiments involving the 88-year-old donor cells. While preliminary data from other studies performed in the same laboratory confirmed that mechanical training increased migration in the 28-year-old group, consistent with previous findings, the trained 88-year-old group exhibited the lowest wound closure rates. In some cases, the wound area even enlarged post-injury. By contrast, the control group of the same age showed improved cell-free gap closure rate.

Age-related cellular changes may explain this discrepancy. Ageing is known to increase cell stiffness and reduce the ability for large, reversible deformations, impairing mechanotransduction compared to younger cells ¹⁶⁰. A study from Schulze et al. (2010) proved that the fibroblast stiffness between two experimental groups with two different ages showed a significant change, as the older cells presented 60% more rigidity in contrast to younger cells ⁹⁶. The same study also reported a decrease in monomeric actin levels as a shift to filamentous actin. The stiffness change in the cell cytoskeleton due to aging can also further affect the cellular response to external stimuli, which can then lead to different gene expression profiles and that, in turn, could explain the contradicting responses of younger and older cells to the same external stress exposure. Additionally, aged cells demonstrate reduced capacity for rapid cytoskeletal reorganization, which could diminish their response to mechanical stimulation. This aligns with findings in murine models, where mechanical loading triggered less transcriptional activation in bones of aged mice versus younger adults ⁹², and cell-free gap closure rates varied more widely in older animals ^{161,162}. Single-cell RNA-sequencing in that

study identified distinct fibroblast subpopulations, suggesting aged tissues may lack the consistent healing pattern seen in younger tissues ¹⁶².

Furthermore, aged dermal fibroblasts produce less collagen types I and III. In vitro comparisons showed decreased cell surface attachment and spreading in older cells, alongside a 68% reduction in type I procollagen content ¹⁶³. This study also identified fewer focal adhesion sites in aged fibroblasts, as visualized via anti-vinculin antibody staining. Additionally, in the unpublished study mentioned earlier, a minor downregulation of COL1A1 by 34% in 88-year-old donor fibroblasts was seen. This decline in ECM production may contribute to delayed wound healing in the older groups.

Since mechanical training introduces substantial stress, a reduction in focal adhesions may lead to increased detachment, apoptosis, and impaired wound closure, potentially explaining why only the trained 88-year-old group was negatively affected. Age-related impairment of wound healing, together with decreased collagen expression, could explain the wound perimeter increase in the older donor control group by the 4-day mark (88 C).

When the skin ages, collagen production is reduced, and the collagen network, on which the fibroblasts are oriented is fragmented and damaged by the increased production of collagen-degrading enzymes ^{95,164}. When comparing the control groups with cells from young or old donors, intuitively it was expected that the gaps in the cell cultures of young donors would close more quickly than those of older donors. However, our investigations did not confirm this. The biggest gap decrease, and the highest gap closure rate (55%) were found for the 88-year-old control group (see Table 13, Figure 36 and 37). Younger organisms are expected to have higher proliferation rates, better secretion of ECM proteins, and, overall, a faster wound closure rate as aging impairs the wound healing pathways and slows down most of the cellular functions ^{164,165}. Nevertheless, a study from 2017, where three age groups (young, middle-aged, and old) were investigated, stated that the in-vivo aged skin fibroblasts do not show gene expression changes for genes COL1A1, TGF β 1, CTGF, and MMP-I in-vitro. Interestingly, COL1A1 expression was lowest in the middle-aged group. The highest collagen content was found in males older than 40 and some subjects younger than 30, while a 65-year-old donor also showed elevated levels, contradicting the widely accepted decline in collagen synthesis with age. Kaisers et al. also demonstrated that gene expression changes in aged skin are not reliably reflected in short-term cultures ⁹³. Furthermore, in the unpublished study mentioned above, a 75% increase in COL1A1 gene expression in the older age group compared to the younger group was also seen. The results presented here are from cells between passages 4–6, even shorter than those used in the Kaisers study. These findings may collectively help explain the unexpected decrease in the gap perimeter observed in the 88-year-old control group (Figures 36 and 37).

Substrate stiffness may also influence fibroblast behaviour ^{8,128}. Migration requires traction forces against the ECM, and fibroblasts are sensitive to substrate deformability. On compliant silicone substrates, cells modified their adhesion structures, morphology, and migration behaviour depending on stiffness ^{3,91,128}. Furthermore, gradients in stiffness can lead to the cells migrating toward softer regions ^{86,91}. Since CellDrums have radial variations in elastic modulus, it is possible that fibroblasts preferentially adhered near the edges, influencing migration

outcomes. Future investigations into how PDMS membrane mechanics affect morphology and adhesion structures would be valuable.

Last but not least, it is important that some caution is given to the interpretation of 2D migration studies. Although the 2D matrices appear to capture important aspects of fibroblast biology relevant for their function in native tissue matrices, it is also clear that specific functions are highly dependent on additional aspects of matrix dimensionality and topography¹⁶⁶. In one migration study, it was shown that placing fibroblasts between two compliant hydrogels, removing the artificial polarity of 2D culture, and providing dorsal and ventral cell adhesions dramatically altered fibroblast morphology and cell-matrix adhesions. Thus, ongoing efforts to study fibroblast migration and function in 3D matrix systems offer important and physiologically relevant insights⁸⁶. Once the 2D migration has been studied, the next step would be to observe the wound healing and migration of fibroblasts in 3D matrices.

To summarize, in this phase of the study, a novel wound creation technique was successfully applied to investigate wound closure rates on the CellDrumTM membranes. Cell lines derived from two donors of different chronological ages were utilized to assess age-related effects. Additionally, equibiaxial tensile stress was introduced as a form of external mechanical stimulation. These two variables were used to establish four experimental groups, each consisting of 10 biological replicates, resulting in a total of 40 samples.

Although the applied steel balls visibly deflected the membranes, a degree of movement across the cell monolayer was still observed, which contributed to slight inconsistencies in the initial gap size. Future optimization could include broader donor age ranges, varied mechanical stimulation parameters, and larger group sizes (>10 replicates) to enhance statistical power. Due to the limited time between cell seeding and gap formation, conventional migration assays were not feasible in this phase. Future studies could incorporate 12-hour FBS starvation prior to steel ball application to enable standard migration assay conditions for comparison.

4.3 Phase 3- Contractility Response Detection using CFA_{refer}

4.3.1 Quality controlled CellDrumTM selection

Each individual CellDrumTM is a physical sensor designed to measure mechanical tensions well below the surface tension of water. Measuring the tensile stress exerted on it by an adhesion-dependent cell monolayer is thus challenging, and this is no different for HAOSMC cell monolayers. This is especially apparent when assessing alterations induced by pharmaceutical agents. It is therefore vital that every effort was to be made to ensure the reliability and reproducibility of the data obtained. Quality control measures had to be conducted for each individual CellDrum sensor. This was essential to ensure the reliability and validity of the results.

Table 15 and 16 present membrane thickness data for CellDrums selected for noradrenaline and ionomycin exposure experiments presented in this section. HAOSMC monolayer membrane thicknesses ranged from 3.56 to 4.00 μm . Those used for the HAOSMC + HAOEC co-culture ranged from 3.38 to 3.83 μm . In both cases, only membranes with a deviation of less than 2% from their average baseline tension (CellDrums without cells) were selected to avoid the use of mechanically unstable and inaccurately functioning membranes.

The data obtained showed high reproducibility and measurement accuracy of the tensile stress, which in turn confirmed that the quality management procedures successfully ensured stable and comparable initial conditions for all experiments.

4.3.2 Phase Contrast Imaging of Cell Morphology

Phase contrast microscopy was used at key culture time points to monitor cell condition and morphology throughout the experiments. Images were captured before seeding, two days after seeding, post-differentiation, and after noradrenaline exposure to assess confluency, attachment, and overall cell health. This ensured consistency and quality across all experimental stages.

The phase contrast microscopy images in Figures 39 and 40 illustrate the morphology and confluency of HAOECs and HAOSMCs before and after seeding on CellDrum™ membranes. Prior to seeding (Figure 39), both cell types display their expected morphology: HAOECs appear cobblestone-like and form a dense monolayer, while HAOSMCs exhibit a more elongated, spindle-shaped structure with moderate spacing. Two days post-seeding on CellDrum™ membranes (Figure 40), both cell types remain viable and well-attached. HAOECs maintain a uniform distribution and start forming junctions, while HAOSMCs spread extensively, preserving their typical morphology and demonstrating good surface adaptation. These observations confirm successful seeding and initial cell adhesion on the CellDrum™ substrates, showing that both cell types were able to adhere to and proliferate on the membranes under standard culture conditions, supporting the membrane's suitability for vascular cell monolayers.

Figure 41 reveals distinct morphological characteristics between the HAOSMC monolayer and the HAOSMC + HAOEC co-culture (right) two days after seeding on CellDrum™ membranes. The HAOSMC monolayer shows elongated, spindle-shaped cells aligned in parallel bundles, indicative of smooth muscle cell phenotype. In contrast, the co-culture displays a denser and more heterogeneous cellular arrangement, with cobblestone-like areas suggestive of endothelial cell integration. These differences support the successful establishment of both mono- and co-culture systems under otherwise identical conditions.

Finally in Figure 42, images comparing HAOSMC monolayers before (left) and after (right) 1000 nM noradrenaline (NA) exposure reveal no significant change cellular appearance. Before treatment, the cells form a uniform monolayer with clear borders and elongated morphology. Post-exposure, subtle changes in cell shape and spacing are visible, but are considered insignificant. The overall integrity of the monolayer appears preserved, suggesting a robust cellular response rather than detachment or damage. The slight reorientation of the cells mentioned previously may be attributed to the nature of the measurement procedure. The CFA_{refer} system measures contractile responses by applying air pressure beneath the membrane, causing a slight membrane upward deflection. As the pressure is released, the membrane returns to its resting state. This repeated deformation over time might resemble a form of biaxial cyclic mechanical loading, potentially inducing subtle alignment of the cells.

Additionally, a localized blurry region can be seen in the right image (Figure 42), which results from the imaging conditions specific to the CellDrum™ platform. Since microscopy was performed with liquid medium present atop the membrane, the natural curvature of the deflected

membrane under pressure creates a slight height gradient across the membrane surface. This variation in focal plane contributes to occasional uneven sharpness in the captured images, particularly toward the center, where deflection is maximal. Despite this, cellular morphology remains clearly discernible, supporting the reliability of both the membrane system and the measurement protocol.

Finally in Figure 43, the phase contrast images of the HAOSMC + HAOEC co-culture before and after exposure to 1000 nM noradrenaline show a stable and confluent monolayer throughout the stimulation period. No major structural changes or signs of cell damage were observed, indicating that the cells remained viable and retained their characteristic morphology. Post-exposure, once more a subtle reorientation of the cells became noticeable, which aligns with expectations given the mechanical measurement setup. This mild shift suggests a physiological response without compromising monolayer integrity.

4.3.3 Vitality Test

The fluorescence microscopy images presented in Figures 44 and 45 serve as a qualitative validation of cell viability prior to NA exposure experiments.

In Figure 45, monolayers of HAOECs (green, left) and HAOSMCs (red, right) display uniform and bright fluorescence, indicating robust viability and healthy morphology. The cells exhibit typical monolayer architecture with clear cytoplasmic staining, confirming that only live cells were stained by the CellTracker dyes. Figure 45 illustrates a co-culture setup in which both cell types are visible, with green-stained HAOECs and red-stained HAOSMCs distributed throughout the field. The mixed staining pattern and overlapping regions further demonstrate successful co-culture integration and cellular vitality. The absence of dim or irregular staining supports that the membranes were seeded with viable, metabolically active cells, ensuring a reliable biological foundation for the upcoming contractile assays.

4.3.4 Fluorescence-Based Cell Characterization by Immunostaining

HAOSMCs were stained with an anti- α -smooth muscle actin (α -SMA) antibody conjugated to Alexa Fluor 488, which selectively labels contractile smooth muscle cells. Since α -SMA is a well-established marker for differentiated HAOSMCs in the contractile phenotype, its detection not only confirmed the cell type but also verified the acquisition of their functional phenotype. HAOEC monolayers were subjected to the same α -SMA staining protocol as a negative control, and as expected, no signal was observed.

Conversely, HAOECs were labelled with an anti-CD31 (PECAM-1) antibody conjugated to Alexa Fluor 647 to confirm endothelial identity. HAOSMCs stained with CD31 as a negative control yielded no detectable fluorescence, further supporting marker specificity.

Figure 46 and 47 reveals the presence of prominent stress fibers and the elongated, aligned morphology that support the differentiation state of the HAOSMCs. The intense fluorescence signal indicates a dense and well-organized filamentous cytoskeleton, confirming the presence of a contractile cell phenotype. Monolayer also showed evenly distributed nuclei across the monolayer, demonstrating a healthy and uniform population on the membrane. In some areas, cells appear layered on top of one another, which is a typical characteristic of smooth muscle cells due to their tendency to form dense, multi-layered structures in vitro¹⁶⁷.

In Figures 48 and 49, the membrane-associated staining pattern confirms the endothelial identity of the cells stained by CD31 antibody, an endothelial junctional marker, and indirectly indicates intact cell-cell junctions, typical for a healthy endothelial monolayer. Once again, the nuclei seem to be homogeneously spread, and the overall cell morphology and spatial organization suggest good viability and adherence. Furthermore, in Figure 50 the distinct spatial separation of the red and green signals confirms the presence of two phenotypically differentiated populations. Furthermore, there was a clear layering of the two cell monolayers on top of each other, with the ECs on top and the SMCs underneath.

Together, the fluorescence staining results provide strong evidence for the phenotypic identity, viability, and structural integrity of both HAOSMCs and HAOECs cultured on the CellDrum™ platform. The presence of marker-specific signals and preserved monolayer organization confirms that cells not only adhered successfully but also maintained their functional characteristics throughout preparation. These findings validate the reliability of the CellDrum™ system in supporting physiologically relevant co-cultures for subsequent mechanical and pharmacological experiments.

4.3.5 Tensile tension of HAOSMC Monolayers

This section discusses the tensile tension response of HAOSMC monolayers cultured on CellDrum™ membranes after exposure to noradrenaline and ionomycin. Tensile tension changes were recorded after each stimulus application, and a dose-response curve was generated to explore potential EC₅₀ values. In addition, statistical analyses were conducted to assess the significance of membrane tension changes at each concentration tested.

As seen in Figure 51 exposure to 50 nM NA resulted in a slight, non-significant reduction in tension compared to control. Thus, this concentration is below the activation threshold for causing a measurable HAOSMC contraction, and may instead reflect early or partial engagement of adrenergic receptors without triggering downstream signalling cascades^{168–170}. At 100 nM NA, a moderate, still non-significant increase in tension was observed.

Statistically significant increments in tensile membrane tension were observed starting at 200 nM NA. This suggests a dose-dependent enhancement of contractile signalling, likely involving a robust rise in intracellular calcium and activation of both calcium-dependent myosin light chain kinase (MLCK) and calcium-independent RhoA/Rho kinase pathways¹⁰¹. These events promote actin stress fiber formation and increased cortical stiffness, hallmarks of active smooth muscle contraction^{6,85}. The NA-induced increase in tensile stress reached saturation levels at approximately 300 nM NA, reflecting near-maximum receptor occupancy and downstream signal transduction. The saturation observed at this point likely corresponds to the full recruitment of contractile machinery and maximal activation of actomyosin dynamics. Increasing NA concentrations further to 400, 500 and 1000 nM did not significantly enhance tension (compared to 300 nM), indicating a plateau phase consistent with receptor saturation and signalling pathway limits¹⁰¹. This plateau suggests that the system has reached its functional contractile limit, beyond which further agonist stimulation yields no additional mechanical output.

The nonlinear regression fitting of the dose–response curve yielded an EC₅₀ of ~100.7 nM with a Hill coefficient of 3.28, suggesting a steep, cooperative activation of contractile response in

HAOSMC monolayers. A slight biphasic effect of NA on membrane tension was observed, as previously described, with a slight reduction at lower concentrations followed by a clear increase at higher doses. This pattern complies with established literature, where β_2 -adrenergic receptor activation is associated with tension reduction at low noradrenaline levels, while α_1 -receptor activation at higher concentrations leads to increased contractility and cellular proliferation^{102,118,120,122,171}. Overall, these findings suggest that the net mechanical response of HAOSMCs is governed by the balance between these opposing adrenergic pathways.

The analysis of ionomycin effect on HAOSMC monolayers demonstrated a clear, concentration-dependent increase in membrane tension, as shown in Figures 52 and 53. Significant elevations in tension were observed at 10 nM ($p = 0.0393$), 100 nM ($p < 0.0001$), and 1 μ M ($p < 0.0001$) compared to the initial control value. The response plateaued between 100 nM and 1 μ M, indicating a saturation of the contractile response. Interestingly, at the highest tested concentration of 10 μ M, membrane tension declined compared to the saturation level, though it remained significantly higher than control ($p = 0.0004$). The fitted sigmoidal dose–response curve ($R^2 = 0.82$) yielded an estimated EC_{50} of 11.44 nM reflecting the high sensitivity of HAOSMC monolayers to calcium influx.

This tensile tension drop at 10 μ M may be attributed to two possible mechanisms. One explanation is the cytotoxicity associated with supraphysiological calcium influx. Supporting this, the study by Høyer-Hansen et al. reported that high concentrations of ionomycin ($\geq 10 \mu$ M) can compromise cell viability and induce membrane damage due to excessive intracellular calcium loading. Such overload may disrupt mitochondrial function and reduce membrane integrity, resulting in a mechanical relaxation. Alternatively, a strong contractile response may have mechanically disrupted the cell–substrate interface, leading to detachment or micro-tears in the cell layer, effectively reducing the transmitted force on the membrane. Both interpretations are consistent with the mechanical readout and known limitations of calcium ionophore exposure^{172,173}.

In summary, the results confirmed a dose-dependent contractile response in HAOSMC monolayers to both NA and ionomycin, with calculated EC_{50} values of ~ 100.7 nM and 11.44 nM, respectively. These values reflect the sensitivity and reactivity of the CFA_{refer}. The instrument seems to have provided a stable and reproducible measurement environment for detecting even subtle mechanical changes as low as 1-2%, highlighting its utility for pharmacological testing. Its sensitivity to both adrenergic and calcium-mediated stimuli, coupled with consistent baseline membrane characteristics and reliable signal detection via CFA_{refer}, underlines the potential of the instrument as a viable system for studying smooth muscle contractility in-vitro.

4.3.6 Tensile tension of HAOSMC-HAOEC Co-cultures

This section discusses the contractile responses of HAOSMC-HAOEC co-cultures exposed to increasing concentrations of noradrenaline and ionomycin. Using the same CFA_{refer} measurement protocol and experimental conditions as for the HAOSMC monolayers, these experiments aimed to assess whether the presence of endothelial cells influences smooth muscle contractility. The co-culture model was used to better mimic the cellular composition of vascular tissue and to investigate potential modulatory effects of endothelial cells on the contractile behaviour of smooth muscle cells.

Given the limited suitability of fitting curve models the data were instead presented as a simple scatter plot with connecting lines to more accurately reflect the observed trends. This allowed the dynamic behaviour of the co-cultures to be conveyed without forcing the data into a predefined model that may not capture the complexity of the biological response.

The results shown in Figure 54 display the contractile responses of HAOSMC-HAOEC co-cultures exposed to increasing concentrations of noradrenaline. Unlike the classic sigmoidal pattern observed in HAOSMC monolayers, the co-culture exhibited a less uniform response profile. A biphasic response of HAOSMC and HAOEC co-cultures to noradrenaline was observed, with an initial decrease in membrane tension at low concentrations (50-500 nM) followed by a partial rebound at 1000 nM. This pattern likely reflects the concentration-dependent engagement of adrenergic receptor subtypes: β_2 -adrenergic receptor activation predominates at low nanomolar concentrations, promoting smooth muscle relaxation via cAMP-mediated inhibition of contractility¹⁷⁴⁻¹⁷⁶. At higher concentrations, α_1 -adrenergic stimulation becomes dominant, reversing the effect and inducing contraction. The relative magnitude of each phase appeared to depend on noradrenaline concentration and endothelial integrity, analogous to how Egashira et al. and Wong et al. observed that the biphasic membrane potential response was dependent on initial membrane potential and was both time- and concentration-dependent^{124,177}. These results highlight the modulatory role of endothelial cells in tuning smooth muscle cell contractility and underline the physiological relevance of the co-culture model in mimicking vascular tone regulation, demonstrating that biphasic α -adrenergic responses represent a fundamental principle across different tissue types and cellular mechanisms.

Moreover, based on the established principle that α -adrenergic agonists can produce biphasic responses in excitable tissues, as demonstrated by Egashira et al. (1980) in guinea pig liver cells where noradrenaline produced initial depolarization followed by hyperpolarization through α -adrenergic mechanisms, it can be hypothesized that in vascular tissue, noradrenaline may similarly produce a biphasic response through sequential activation of different receptor populations and cellular mechanisms¹⁷⁷. This hypothesis is further substantiated by observations with other α_2 -adrenoceptor agonists, such as dexmedetomidine, which has been shown to elicit similar concentration-dependent biphasic effects in isolated, thus illustrating the potential for complex vascular responses from related adrenergic compounds¹²⁴.

Notably, the presence of endothelial cells may modulate this response through the release of nitric oxide or other paracrine signals, dampening excessive contractile responses and contributing to vascular tone regulation^{100,123,178}. Our findings align with previous reports of receptor crosstalk and concentration-specific behaviour in vascular tissues and support the relevance of using co-culture systems to more closely mimic in vivo vascular physiology.

For the evaluation of ionomycin-induced contractile responses in the HAOSMC-HAOEC co-culture model, the data revealed once again a non-linear pattern that did not follow typical sigmoidal or biphasic dose-response behaviour. An initial rise in membrane tension at lower concentrations was followed by a decrease and subsequent partial rebound at higher doses, suggesting a complex interplay of cellular responses. Given this variability and the limited sample size ($n = 2$), standard curve-fitting approaches could not be reliably applied. Therefore,

the results were represented using a line graph and bar chart format to show overall trends without imposing a pre-defined model, similar to the NA measurements.

As depicted in Figures 55 and 56, a sharp initial increase in membrane tension occurred between the control and 1 nM ionomycin ($p=0.0042$), followed by a further rise at 10 nM ($p=0.0002$), marking the peak contractile response. Beyond this point, the tension declined at 100 nM and reached a lower level at 1 μ M ($p=0.0164$), suggesting a transient desensitization or counter-regulatory effect. Interestingly, a secondary increase in tension was recorded at 10 μ M ($p=0.0005$), forming a non-monotonic, biphasic response curve. These nonlinear effects may reflect a dual mechanism: low-dose stimulation promotes physiological calcium signalling and contraction, while higher concentrations may transiently impair contractility due to calcium overload or desensitization. The observed partial recovery at 10 μ M could result from compensatory mechanisms or changes in cytoskeletal tension regulation influenced by the endothelial layer.

The sustained contractile response at 10 μ M ionomycin, despite the preceding decline, may be attributed to the protective effects of the co-culture architecture. Co-culture models of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) have demonstrated significant co-protective functions, leading to enhanced cellular resilience against harmful stimuli. For instance, in direct co-culture, ECs displayed reduced sensitivity to inflammatory agents, evidenced by decreased expression of adhesion molecules in response to TNF- α , along with an increase in protective factors ¹¹⁵. This reduced sensitivity to inflammatory or toxic agents, as highlighted by the more physiologically representative nature of co-culture systems compared to single-cell cultures, suggests that such models provide cells with an environment that can minimize direct cytotoxic effects (Truskey et al., 2010) ¹¹⁶. This protective mechanism, absent in monocultures, could also explain the unique biphasic response pattern observed in the co-culture system both for ionomycin and noradrenaline. It is also possible that interactions between endothelial and smooth muscle cells can modulate calcium dynamics and responses to cytotoxic influences compared to monocultures, contributing to observed variability. For instance, voltage-dependent calcium entry in smooth muscle cells can trigger intricate calcium signalling in adjacent endothelial cells, leading to a feedback mechanism that modulates vascular tone ¹⁷⁹. This intercellular communication, alongside the demonstrated reduction in endothelial cell activation by inflammatory cytokines like TNF- α in co-culture (Wallace et al. 2010), further highlights how these integrated cellular environments provide a more nuanced and potentially protective response to injurious agents than isolated cell systems (Truskey et al., 2010).

Furthermore, the tension values recorded in this co-culture setup were consistently higher than those in the SMC monoculture, possibly reflecting the amplifying role of endothelial-derived signals or enhanced matrix anchoring in the dual-layer system ¹⁸⁰. Endothelial cells can modulate smooth muscle tone via paracrine factors such as nitric oxide or endothelin, but these may also shape cytoskeletal responses during pharmacological stimulation. Our data show that ionomycin triggers a strong, concentration-sensitive contractile response in HAOSMC-HAOEC co-cultures, with peak tension at low nanomolar doses. These findings highlight the importance of cellular context in calcium-mediated mechanotransduction and underscore the utility of co-culture models for studying vascular contractility in more physiologically relevant environments.

However, it is important to mention that in an unpublished study from our laboratory in FH Aachen, using the same cell lines in older passage numbers (passage 4 for both cell lines), despite showing highly similar results in HAOSMC monolayer samples, showed a significant and unmistakable contraction in membrane tension. All experimental conditions were kept identical besides the passage number of the used cells. Although the cell seeding is thought to be performed equally, errors in pipetting, cell counting and sample handling can also cause differences in cell confluency. The above mentioned discrepancy in the contractile NA response in cells with earlier passages highlights that cellular responses in-vitro can be highly dependent on passage number, potentially due to altered receptor sensitivity or activity, which in turn influences overall cellular function. A relevant example of this phenomenon is seen in a study by Nakaki et al. on the effects of catecholamines on DNA synthesis in vascular smooth muscle cells (VSMC). This study showed that the dose-response curves for NA are biphasic in lower passage cells, demonstrating both stimulatory (via α_1 -adrenergic receptors) and inhibitory (via β_2 -adrenergic receptors) effects depending on concentration and confluency. However, higher passage cells exhibited only inhibitory effects, suggesting a shift in adrenergic receptor-mediated signalling pathways with increased passage number¹⁷¹. This exemplifies how changes in cellular characteristics over prolonged in vitro culture can lead to different functional outcomes, which may account for the changes in the higher passage co-culture samples in our unpublished study.

To sum it all up Norepinephrine triggered a pronounced sigmoidal increase in tensile tension within HAOSMC monolayers, elevating contractile force by 16.9% above control levels (EC_{50} : 100.70 nM). When these smooth muscle cells were co-cultured with endothelial cells, however, the response completely reversed, norepinephrine now inducing a 12.6% relaxation compared to control (EC_{50} : not applicable). Ionomycin demonstrated contrasting behaviour. In isolated smooth muscle monolayers, this calcium ionophore produced a modest 10.9% tension increase (EC_{50} : ~11.44 nM). In co-culture conditions, however, its contractile effect was significantly amplified, generating tensions 61% higher than control with enhanced sensitivity (EC_{50} : <1 nM). These differential responses demonstrate that cellular context fundamentally alters pharmacological activity. Both compounds exhibited their expected behaviour in their respective experimental conditions. In isolated smooth muscle cells, direct receptor-mediated responses predominated, while in co-culture, the norepinephrine-induced relaxation represents the anticipated outcome of α_2 -adrenoceptor activation on endothelial cells, triggering nitric oxide release and subsequent vasodilation that overrides direct smooth muscle contraction.

Even with strict quality control steps, every CellDrum, manually produced PDMS silicone membranes with a thickness range of 3.4-4 micrometers exhibits differing pre-tension due to minor variations in the fabrication process. Although only CellDrums with stable and low-variance tension values are selected for tensile tension measurements, small differences in control tension remain between individual membranes. To remove these differences and isolate the true effect of noradrenaline or other test agents, an offset correction is performed after measurements.

The offset correction requires a brief explanation: even if the CellDrum membranes adhere perfectly to the applicators without wrinkles or leaks, even if their thickness has been precisely measured interferometrically and included in the tension calculation, a mechanical pretension remains in the membranes that is invisible. This causes a more pre-stressed membrane to bend

less with the same contraction of the cells, while the less pre-stressed membrane bends more (comparable with membranes of different thicknesses). However, the variability of the technical repeats is negligible. This means that if the same membrane is measured n times in succession, the result is very reliable. An offset correction was performed for all tensile tension data.

As mentioned along with the results of a previous unpublished study, experimental parameters such as cell passage number and confluency are critical in determining adrenergic receptor profiles in VSMCs. Passage number influences the relative abundance and responsiveness of adrenergic receptors, with lower-passage cells maintaining a balance between α_1 and β_2 receptor-mediated pathways, while higher-passage cells often shift toward dominant β_2 -mediated responses. Similarly, cell confluency affects receptor expression, where sub confluent cells may exhibit stronger α_1 -receptor activity, while confluent cells display a relative increase in β_2 -mediated responses. These shifts suggest that experimental consistency in passage number and confluency is essential, as variations can directly alter receptor expression patterns and responsiveness to catecholamines, impacting the interpretation of adrenergic signalling in vascular smooth muscle studies¹⁷¹.

For future subsequent measurements, it is imperative that only primary and human cells are utilised. The following two points provide the rationale behind this. Firstly, it has been demonstrated that there are distinct disparities in receptor ratios between animal and human endothelial cells. Secondly, endothelial cells appear to undergo a loss of functionality of nitric oxide (NO) production, which declines further with the number of cell passages during culturing. Consequently, judging by the results of our tensile tension measurements, the passage number must not exceed 3.

Finally, the contractile responses of the co-culture system demonstrated that even small, non-linear changes in membrane tension could possibly be detected using the CellDrumTM platform and CFA_{refer} measurement system. Both noradrenaline and ionomycin displayed statistically significant alterations in membrane mechanics, despite the added complexity introduced by endothelial cell interactions. These results confirm that the co-culture setup retains functional contractility and remains responsive to pharmacological stimulation. Moreover, the ability to monitor these changes in real time through tension-based readouts highlights the sensitivity and suitability of the CellDrumTM system for investigating dynamic cell–cell interactions and compound effects in vascular-like constructs. This positions the platform as a promising tool for future studies in vascular physiology, pharmacological screening, and even disease modelling under controlled mechanical conditions.

5. Conclusion

In this thesis the CellDrum™ platform was successfully tested across very different experiments, each targeting a differing aspect of its functionality and application potential in cell-based biomechanical assays.

In the first phase, membrane production was optimized and standardized, focusing on understanding how PDMS volume and ambient temperature influenced membrane thickness. While the relationship between temperature and membrane thickness was subtle, the controlled production process ensured low variance and reproducibility. Mechanical tests confirmed that small variations in thickness did not actually affect membrane deflection or compliance, supporting the selection of membranes within a reliable range for experimental use. The quality control workflow which combines visual pre-inspection, thickness measurements, and a baseline mechanical testing, proved effective in identifying suitable CellDrums for further experimental applications. Despite the successful testing of an alternative material for the CellDrum™ applicators, the switch to this new material was found not crucial for the time being. This decision was driven by two primary considerations. Firstly, it was to maintain consistency and comparability with existing CellDrum™ studies conducted in our laboratory, since all previous experimental data was generated while using the original material. Secondly, the motivation for finding alternative materials was actually related to potential future commercialization of the CellDrum™ technology, which is outside the scope of this thesis. However, since the data still showed relevant information, the results were still included.

The second phase centered on applying the CellDrum™ platform to study wound healing dynamics in a dermal fibroblasts derived CellDrum™ based assay from young and aged donors. By creating standardized circular “wounds” in the cultured fibroblast layers and monitoring this gap closure over time, the system enabled the detection of age-related differences in cell migration and the impact of mechanical stimulation. Notably, mechanical training accelerated wound closure in the younger donor group and produced varied responses in aged fibroblasts, some of which showed impaired healing or even expansion of the wound area. These outcomes highlight CellDrum™’s ability to sensitively capture donor-specific and condition-dependent differences in regenerative behaviour, confirming its relevance in aging research and mechanobiology.

In the third and so far, final phase, cell contractility studies were performed using HAOSMC monolayers and HAOSMC-HAOEC co-cultures. The previously mentioned CFA_{refer} system successfully captured membrane tension responses to increasing concentrations of noradrenaline and ionomycin. In smooth muscle monolayers, dose–response relationships exhibited clear patterns, including a mild biphasic effect of noradrenaline and a concentration-dependent contractile response to ionomycin, both yielding quantifiable EC₅₀ values. Co-culture experiments presented more complex contractile responses, likely influenced by endothelial modulation. Although standard pharmacodynamic curve fitting was not suitable for the co-culture data, significant changes in membrane tension were still measurable, suggesting the presence of interactive signalling between the two cell types. These results demonstrated that the CellDrum™ system can stay effective even in more complex, physiologically relevant cell configurations.

Although this is not the subject of this thesis, we would like to conclude by mentioning that the CFA_{refer} platform also has great potential for applications with self-exciting hIPS-derived cardiomyocytes, where its ability to monitor contraction force on ultra-thin membranes can be used to study cardiac cell rhythm, force generation, and pharmacological responsiveness. The feasibility and relevance of this application have already been recognized through a US patent received in 2021 by Artmann G. et al, (EU Patent in 2025) underscoring the translational value of extending the CellDrum™ system into cardiovascular research¹⁸¹.

Moving forward, this technology could potentially offer significant potential for expansion into more advanced in vitro models. Incorporating ECM components such as collagen gels or Matrigel could facilitate the creation of three-dimensional cultures that better mimic native tissue environments as for example regular vessel walls. Expanding co-culture systems to include other cell types like immune or stromal cell populations could actually provide deeper insights into multicellular signalling under mechanical influence. Furthermore, implementing cardiomyocyte models within the CellDrum™ platform would open additional avenues in cardiac disease modelling, cardiotoxicity screening, and regenerative cardiac medicine.

The CellDrum™ platform shows futuristic promise for studies in the field of drug screening, particularly for compounds affecting cell mechanobiology like contractility or migration, and even for personalized medicine, allowing the study of patient-derived cells under specific mechanical conditions. This technology can also be used in regenerative medicine, allowing for optimized scaffold design, and in toxicology, by investigating the mechanical impact of various substances on cell health.

In summary, the CellDrum technology, in combination with the CFA_{refer}, now offers a compelling alternative to vascular rings and organ explants, like the Langendorff heart, which are used for similar indications but are considered questionable for obvious reasons. We are convinced that the CellDrum™ system has proven to be a sensitive, reproducible, and versatile platform appropriate for a broad range of biomechanical and cellular investigations.

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List of Abbreviations

α -SMA: Alpha-Smooth Muscle Actin

α 1-AR: Alpha-1 Adrenoceptor

α 2-AR: Alpha-2 Adrenoceptor

β 2-AR: Beta-2 Adrenoceptor

AF: Alexa Fluor

AFM: Atomic Force Microscopy

bFGF: Basic Fibroblast Growth Factor

CFArefer: Cell Force Analyzer (measurement system)

CMFDA: 5-Chloromethylfluorescein Diacetate (CellTracker Green)

COL1A1: Type I Collagen

DAMPs: Damage-Associated Molecular Patterns

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl Sulfoxide

DPBS: Dulbecco's Phosphate Buffered Saline

EC: Endothelial Cell

ECM: Extracellular Matrix

eNOS: Endothelial Nitric Oxide Synthase

FBS: Fetal Bovine Serum

FoW: Floating-on-Water

HAOEC: Human Aortic Endothelial Cells

HAOSMC: Human Aortic Smooth Muscle Cells

IL: Interleukin

mRNA: Messenger Ribonucleic Acid

MAPK: Mitogen-Activated Protein Kinase

MMPs: Matrix Metalloproteinases

NA: Noradrenaline

NO: Nitric Oxide

PDGF: Platelet-Derived Growth Factor

PDMS: Polydimethylsiloxane

PEEK: Polyetheretherketone

PEG: Polyethylene Glycol

RNA: Ribonucleic Acid

ROCK: Rho-associated Protein Kinase

SMC: Smooth Muscle Cell

SOP: Standard Operating Protocol

TFM: Traction Force Microscopy

TGF- β : Transforming Growth Factor-beta

TIMPs: Tissue Inhibitors of Metalloproteinases

TLRs: Toll-like Receptors

TNF- α : Tumor Necrosis Factor-alpha

TRP: Transient Receptor Potential (channels)

UV: Ultraviolet

VEGF: Vascular Endothelial Growth Factor

VSMC: Vascular Smooth Muscle Cells

YAP/TAZ: Yes-associated Protein / Transcriptional Coactivator with PDZ-binding motif

Acknowledgement

I would like to express my deepest gratitude to my supervisors, Professor Nicole Teusch and Professor Aysegül Temiz-Artmann, for their invaluable guidance, continuous support, and encouragement throughout the course of my doctoral studies. Their expertise, insightful feedback, and generous availability have been instrumental in shaping this work and in fostering my development as a researcher. I am sincerely thankful for their patience, constructive criticism, and the fruitful collaboration that greatly contributed to the successful completion of this project. I would also like to sincerely thank Professor Gerhard M. Artmann for his advice, generosity, and constant willingness to share his knowledge, which greatly enriched my academic experience.

I am deeply thankful to all my colleagues in the laboratory for creating a stimulating and friendly working environment. I am especially grateful for their encouragement during challenging experiments, their constructive feedback on my ideas, and the countless moments of teamwork that made even the most difficult tasks manageable. The support, collegiality, and sense of community in the lab have not only contributed to the success of this work but have also made my time in the lab truly enjoyable and memorable.

Words cannot fully capture the depth of my gratitude to my mother, who has been my greatest source of support throughout my academic journey. Her encouragement, guidance, and sacrifices, both financial and emotional, have enabled me to pursue my studies and reach this milestone. From an early age, I was inspired by her love for learning, her tireless work ethic, and her remarkable resilience in raising me. The curiosity, determination, and passion for knowledge that have carried me through my doctorate are actually the result of the values and example she set for me. I would also like to thank my father for always being there for me and for his steady support along the way. His down-to-earth attitude, sense of humour, and strong work ethic have kept me grounded and reminded me of the value of persistence and balance in life. Finally, I want to express my heartfelt thanks to my significant other, whose patience, kindness, and encouragement have been invaluable. His ability to bring calm during stressful times and his willingness to support me meant more to me than words can convey. His care and belief in me made this experience both more manageable and more meaningful.

To all of you, thank you for standing by me and for believing in me every step of the way. Every experiment, every challenge, and every late night has been a small step toward understanding, both the world and myself.

Publications

Parts of this thesis have already been published in international peer-reviewed journals.

Publications in international peer-reviewed journals:

- I. Uysal, K.;** Creutz, T.; Firat, I.S.; Artmann, G.M.; Teusch, N.; Temiz Artmann, A. Bio-Functionalized Ultra-Thin, Large-Area and Waterproof Silicone Membranes for Biomechanical Cellular Loading and Compliance Experiments. *Polymers* **2022**, *14*, 2213. <https://doi.org/10.3390/polym14112213>

Roles: K.U. contributed to the conceptualization, methodology, validation, formal analysis, investigation, writing- original draft preparation, writing- review and editing, visualization, and project administration of the manuscript.

- II. Uysal, K.;** Firat, I.S.; Creutz, T.; Aydin, I.C.; Artmann, G.M.; Teusch, N.; Temiz Artmann, A. A Novel In Vitro Wound Healing Assay Using Free-Standing, Ultra-Thin PDMS Membranes. *Membranes* **2023**, *13*, 22. <https://doi.org/10.3390/membranes13010022>

Roles: K.U. contributed to the conceptualization, methodology, validation, formal analysis, investigation, writing- original draft preparation, writing- review and editing, and visualization of the manuscript.

Eidesstattliche Erklärung

Hiermit versichere ich an Eides statt, dass die vorgelegte Dissertation mit dem Titel:

„Development and Standardization of the CellDrum™: A Biofunctionalized Ultra-Thin PDMS Membrane Sensor for Advanced Cell Culture and Mechanobiological Testing”

von mir selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der Grundsätze zur Sicherung guter wissenschaftlicher Praxis, kooperierend mit der Heinrich-Heine-Universität Düsseldorf an der Fachhochschule Aachen erstellt worden ist. Die Dissertation wurde in der vorgelegten oder in ähnlicher Form noch bei keiner anderen Institution eingereicht. Ich habe bisher keinen erfolglosen Promotionsversuch unternommen. Die von mir vorgelegte Dissertation ist von Prof. Dr. Teusch & Prof. Dr. Temiz-Artmann betreut worden.

Jülich, 24/09/2025

Uysal, Karya