Trends in Biotechnology

CellPress OPEN ACCESS

Review

Cell-stretching devices: advances and challenges in biomedical research and live-cell imaging

Iordania Constantinou[1,2,5,*] and Effie E. Bastounis[3,4,5,*]

Basic human functions such as breathing and digestion require mechanical stretching of cells and tissues. However, when it comes to laboratory experiments, the mechanical stretching that cells experience in the body is not often replicated, limiting the biomimetic nature of the studies and the relevance of results. Herein, we establish the importance of mechanical stretching during *in vitro* investigations by reviewing seminal works performed using cell-stretching platforms, highlighting important outcomes of these works as well as the engineering characteristics of the platforms used. Emphasis is placed on the compatibility of cell-stretching devices (CSDs) with live-cell imaging as well as their limitations and on the research advancements that could arise from live-cell imaging performed during cell stretching.

Highlights

Mechanical stretching is experienced ubiquitously by human tissues and their constituent cells and impacts biochemical and (mechano)biological processes relevant to health and disease.

Numerous cell-stretching devices (CSDs) have been developed and used to apply mechanical strain to cells and tissues in *in vitro* settings, however, few have shown compatibility with live-cell microscopy.

In most microscopy-compatible CSDs, imaging is performed post-mechanical stretching while live-cell imaging during cyclic stretching is very rarely shown, especially at high stretching frequencies and for long experiments (i.e., hours long).

Live-cell imaging during cell stretching has revealed distinct time-dependent biomechanical features of cells, such as cell fluidization at high amplitudes or frequencies, or cracking followed by recovery of cell monolayers.

Integrating cell-stretching in biomedical research

Mammalian cells *in vivo* are constantly sensing and responding to the mechanical forces originating from adjacent cells, their environment, and to the changing properties of their **extracellular matrix (ECM)** (see Glossary) [1,2]. Many physiological functions such as breathing, digestion, muscle contraction, heartbeat, and brain development entail cells being constantly stretched and/or compressed. A central element in these processes is the ability of cells to sense these mechanical forces ('mechanosensing') and transduce mechanical information as a response ('mechanotransduction') (Box 1). For example, cells can actively probe the stiffness of the ECM on which they are anchored by stretching it, that is, by exerting traction forces on it via cell–ECM adhesion complexes [3], though not all probing mechanisms that allow cells to sense ECM mechanical properties have been elucidated. Mechano-sensation and -transduction of information produces a set of biochemical and biomechanical responses that reprogram cell changing cellular processes, such as motility and lineage differentiation, thus critically impacting human (patho)physiology [4–6] (Figure 1, Key figure).

Since mechanical stretch (MS) is ubiquitous in tissues and constituent cells, it is critical to account for it during experimentation *in vitro*. To uncover the spatiotemporal alterations that occur at the cellular and molecular scale during MS, CSDs are utilized that allow application of MS to multicellular assemblies. Despite some compatibility with microscopy, long-term imaging of live cells during MS is still limited. In this review, we first highlight the importance of further developing CSDs for biological and biomedical applications by providing background related to the importance of MS in health and disease. We then delve into the mode of action of commonly used CSDs, their limitations, applicability, and compatibility with live-cell videomicroscopy and further highlight the discoveries that have emerged in basic cell biology and beyond. Finally, we discuss open questions and ways to improve biomedical appeal and applicability of CSDs. We hope that with this critical

[1]Institute of Microtechnology (IMT), Technische Universität Braunschweig, Alte Salzdahlumer Str. 203, 38124 Braunschweig, Germany
[2]Center of Pharmaceutical Engineering (PVZ), Technische Universität Braunschweig, Franz-Liszt-Str. 35a, 38106 Braunschweig, Germany
[3]Institute of Microbiology and Infection Medicine (IMIT), Eberhard Karls University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany
[4]Cluster of Excellence "Controlling Microbes to Fight Infections" EXC 2124, Eberhard Karls University of Tübingen, Auf der Morgenstelle 28, 72076 Tübingen, Germany

Box 1. Mechanosensing and mechanotransduction

The biomechanics of many cellular processes, including cell motility and anchorage to the ECM, are determined by the cytoskeleton, a biopolymer network of actin filaments, microtubules, and intermediate filaments that span the entire cell, determine cell shape, and provide adherent cells with mechanical strength [59]. Among the many material properties of the cytoskeleton, most striking is its ability to be autonomously and actively reorganized by cells in response to changes in the physical properties of their extracellular environment [e.g., ECM stiffness or mechanical stretching (MS)]. At the heart of this mechanical adaptability is a set of biochemical and mechanical processes by which cells sense the geometry of their environment and physical forces exerted on their environment through focal adhesions, a process referred to as 'mechanosensing' [59,60]. Cells have a multitude of distinct receptors on their surface that are thought to be mechanosensing elements able to transduce mechanical information intracellularly, leading to activation of distinct biochemical signaling pathways. For instance, MS is sensed by cells through stretch activated proteins, like integrins, which act as anchorage points allowing cells to adhere to their ECM and transduce forces onto it [3]. This activates calcium influx in response to MS along with various kinases that ultimately reorganize the cytoskeleton and its ability to generate forces and contractility. This is in part achieved due to the force-generating capability of molecular motors (myosin II), which slide along actin filaments past one another to generate contractility, while actin actively polymerizes in a polar fashion. The motor activity of myosin II is elicited through a power stroke mechanism fueled by ATP hydrolysis. Using TFM, one can measure the forces that cells exert on their environment, which can be thought as a proxy of how well focal adhesions are organized and connected to the underlying cytoskeleton [61,62]. Understanding the underlying design principles of the cellular machinery, its adaptable networks, and its various responses to extracellular forces is thus of outmost importance, not only for understanding how the cell works in health and disease but also for better designing engineered tissues and for regenerative medicine. Depending on the research question posed, the time scales of interest can vary from seconds to minutes (e.g., dynamics of cellular adhesions or changes in cell stiffness upon acute static MS application [46,63]) to hours or even days (e.g., cytoskeletal and traction force alterations in response to infection of mammalian cells by intracellular bacterial pathogens [64]).

review we will inspire further developments in CSDs to allow long-term imaging during stretching, to more readily explore the role of MS in modulating cell and tissue **(mechano)biology**.

MS in tissues and their constituent cells in health and disease

Various tissues and their constituent cells are exposed to MS [3,7]. Changes in physiological MS can be either the cause or the consequence of pathologies (Box 2). For example, in the myocardium, MS guides the development and regulation of periodic heart contractions, while misregulation of cardiomyocyte response to stretch is linked to various cardiac diseases [4] (Box 2). Not surprisingly, under developmental conditions MS guides stem cell differentiation *in vitro*, while in regenerative medicine MS improves strength and functionality of engineered myocardial tissues [8]. Interestingly, resident macrophages can also sense MS thanks to their interactions with neighboring cardiomyocytes. Imaging revealed that macrophages become activated upon MS, thus protecting the failing heart through promotion of cardiac remodeling [9]. Similarly, in the intestine MS can reprogram intestinal epithelial cells (IECs) altering their gene expression [10] and generating satiety signals for feeding regulation [11] (Box 2). Imaging of IECs after exposure to MS revealed that the latter allows coculture of IECs with commensal microbes for weeks, something difficult in static cultures due to bacterial overgrowth causing IEC death [12,13]. Consistently, patients with irritable bowel disease, where MS is impaired, experienced bacterial overgrowth [14]. During infection, the presence of MS and coculture with commensals protects the epithelium against infection and injury [12]. However, the precise contributions of individual species and the mechanisms they employ to regulate infection was not assessed, possibly due to incompatibility with videomicroscopy. Decoupling the effect of extracellular physical cues from that of the microbiome could allow unequivocal determination of their contributions. (See Box 3.)

Airway epithelial cells (AECs) also experience MS during breathing. Stretch-induced **strain** is an order of magnitude higher in the presence of pathologies such as asthma-induced bronchospasm [15], while acutely applied static (as in lung injury) versus chronic cyclic MS has distinct effects on AEC functions and fate [16] (Box 2). This underlines the importance of CSDs that allow independent tuning of parameters such as stretch magnitude (induced strain), frequency, and

[5]These authors contributed equally to this work

*Correspondence:
i.constantinou@tu-braunschweig.de
(I. Constantinou) and
effie.bastounis@uni-tuebingen.de
(E.E. Bastounis).

CellPress
OPEN ACCESS

Key figure

Mechanical stretch (MS) can alter many aspects of cellular behavior and function

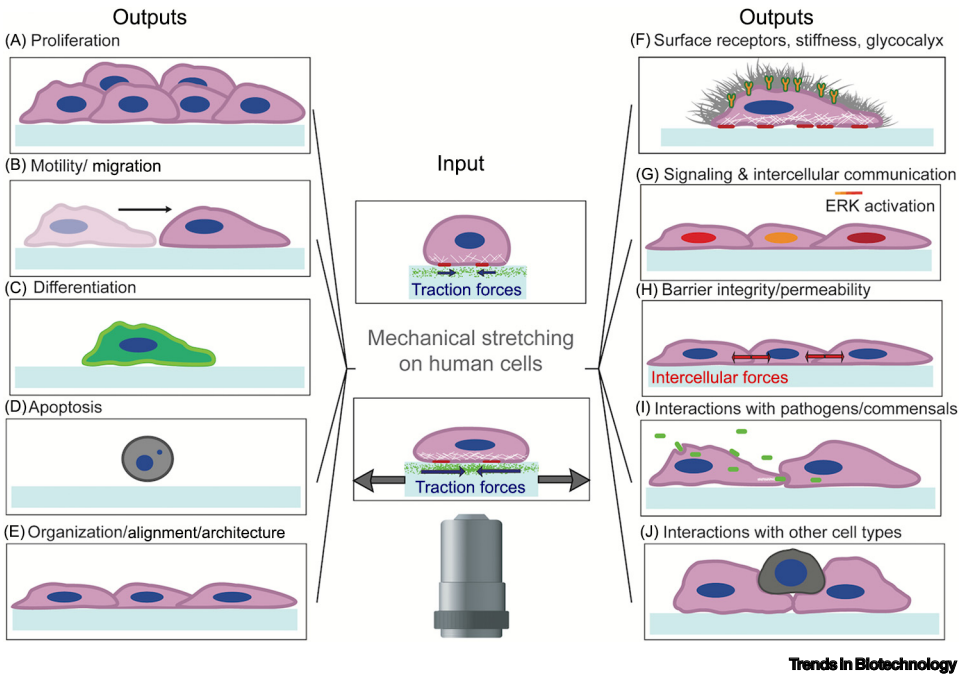

Figure 1. (Middle) Illustration shows a cell residing on a deformable flat matrix (middle) embedded with tracer beads onto which the cell exerts forces, which can be assessed by traction force microscopy [61]. When the cell is stretched statically at a certain strain, cytoskeletal reinforcement occurs, the cell spreads (changes its shape) and exerts increased traction forces onto its matrix via its reorganized cytoskeleton [30,40]. Additional cell functions and behaviors that can change during static versus cyclic MS applied on cells include (A) proliferation [69,70]; (B) cellular migration [36]; (C) lineage differentiation [24,71]; (D) apoptosis [51]; (E) cellular alignment and changes in 3D organization; (F) cortical stiffness, amount and composition of surface receptors, glycocalyx organization; (G) intercellular communication for cells in monolayer and signaling dynamics; (H) barrier integrity and intercellular force transduction; (I) interactions with pathogens, including with commensal microbes; and (J) interactions with other cell types, including with immune or cancer cell lines. All these changes could, in principle, be monitored via videomicroscopy. Abbreviations: ERK, extracellular signal-regulated kinase.

direction. Similar to AECs, endothelial cells (ECs) lining the inner lumen of blood vessels elongate perpendicular to the MS direction and alter cell–ECM and cell–cell adhesions in a strain magnitude- and time-dependent manner [17,18]. Exposure to high magnitude (18%, as during bronchospasm of asthmatic patients) as compared with physiological strain (5%) compromises endothelial **barrier integrity** as a result of the activation of **extracellular signal-regulated kinase (ERK)** signaling and changes in cell contractility that also differ when MS is statically versus cyclically applied to cells [18] (Figure 1). High magnitude, as compared with physiological strain, can also alter the transcription profile of ECs and their glycocalyx (protective sugar-sieve coating cells) and induce ECM remodeling, which can contribute to atherosclerotic plaque formation [19]. Investigating the driving mechanisms in real time would allow a better understanding of how blood vessels get damaged and how atherosclerosis develops. MS also possibly affects the dynamics of vesicular trafficking processes, including endocytosis, and the subsequent increase in cell membrane tension [20,21]. Interestingly, a recent *in vivo* study showed that endocytic

Glossary

Actin: important cytoskeletal protein that (de)polymerizes to form long, dynamic filaments within cells, supporting cell shape and force transduction, which are essential for example for cell motility.
Actuation: refers to actuators, part of a device or system that helps it to achieve physical movement by converting energy into mechanical force.
Barrier integrity: mechanism of regulation of intercellular adhesions for cells in monolayer, which allows the permeability of, for example, essential ions, nutrients, and water, but restricts the entry of insults (e.g., pathogens).
Cell-stretching platform: complete cell-stretching system that includes a CSD as well as all other related hardware and software necessary to execute a cell-stretching experiment.
Elastomeric materials: polymers that display rubber-like elasticity, are dimensionally stable, but elastically deformable plastics with low Young's modulus.
Extracellular matrix (ECM): large network of proteins and other molecules that surround, support, and give structure to cells and tissues in the body.
Extracellular signal-regulated kinase (ERK): stretch-sensitive protein kinase that, upon activation, regulates cell processes such as proliferation, differentiation, migration, and cell cycle.
Focal adhesions: complexes located at the basal side of cells that allow anchorage to the ECM and are largely composed of transmembrane heterodimeric proteins named integrins.
Förster resonance energy transfer (FRET): the transfer of energy from a fluorescent donor in the excited state to an acceptor in the ground state, which can be quantified via FRET microscopy by using appropriate sensors.
Hysteresis: a change in the state of a system that depends not only on current inputs but also on past inputs (i.e., its history).
Inverted microscope: inverted or upright microscopes have objectives below or above the stage holding the sample, respectively (i.e., the sample is imaged from the bottom or the top, respectively).
Mechanobiology: field at the interface of biology and mechanics, investigating the crosstalk between biological and mechanical properties of cells and tissues and how those regulate cell and tissue functions.

Box 2. MS of tissues and constituent cells in health and disease

MS during heart beating

Cardiomyocytes, contractile involuntary striated muscle cells, are one of the major cell types of the myocardium that enable coordinated heart contraction through their electromechanical connections. In diseases such as chronic hypertension, in order to maintain cardiac output in response to increased workload and compensate for inefficient blood pumping out of the heart, cardiac hypertrophy develops, associated with myocardial remodeling, increased cardiomyocyte size, and increased magnitude of stretching (Figure I) [65].

MS during intestinal peristalsis

Intestinal MS resulting from peristalsis is experienced by IECs lining the inner lumen of the intestinal wall. In inflammatory bowel diseases, such as Crohn's disease and ulcerative colitis, MS is compromised (Figure I) [12], impacting the microbiome composition and IEC susceptibility to infection due to IEC reprogramming, including alterations in gene expression [10]. This highlights the importance of exposing *in vitro* cells to MS to more closely emulate their *in vivo* status. The IEC barrier integrity and 3D architecture also more closely mimic those of *in vivo* tissues when MS is applied [12].

MS during respiration

Bronchial epithelial cells (BECs) in the lung are cyclically subjected to 5–10% stretch-induced strain during physiological breathing conditions [15]. However, in patients with acute respiratory failure who receive mechanical ventilation, strain levels can increase to 15–25%, which can aggravate pre-existing conditions (Figure I). Under normal conditions, BECs form a physical barrier comprised of robust cell–cell adhesions, which protect pulmonary airways from inhaled irritants and pathogens [66]. Allergens and pathogens can induce constriction of the airways, exposing the epithelium to up to an order of magnitude higher strain [67], inducing structural, biophysical, and molecular changes in the epithelium that can lead to remodeling of the ECM and loss of barrier integrity [68].

MS during blood pumping in blood vessels

ECs form a single layer lining the inner lumen of blood vessels thus forming a protective barrier separating circulating blood from surrounding tissues. Highly mechanosensitive ECs [17] respond to alterations in the cyclic MS they experience due to blood pumping, changing their proliferation and barrier integrity (Figure I) [69]. In tissue engineering, priming stem cells to MS leads to tissue-engineered vascular grafts with higher mechanical strength and better function following implantation into animals [24].

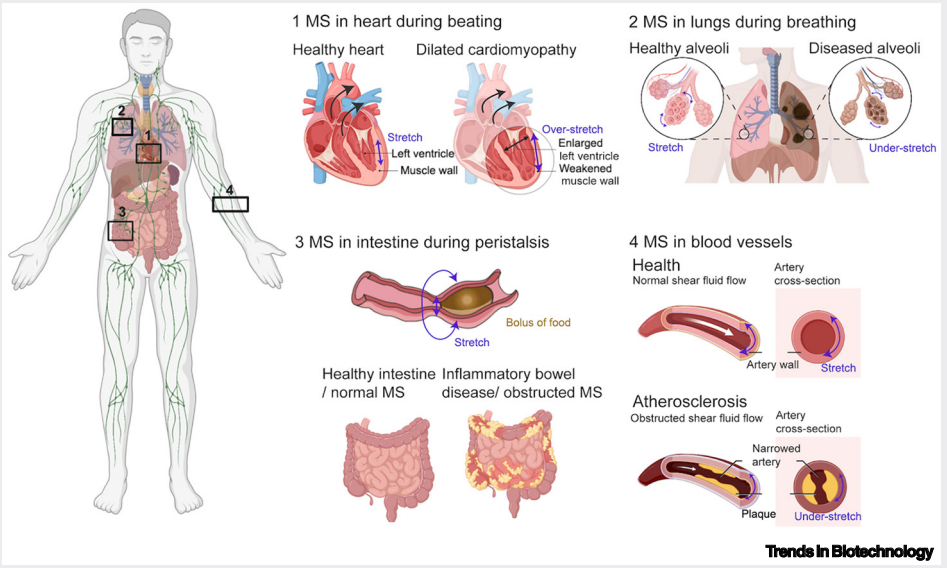

Figure I. Illustration of human tissues that experience differential mechanical stretch (MS) in health and disease. See Box 2 text for a detailed explanation.

Motor actuation: conversion of electrical signals into movement using a motor.
Myosin II: molecular motor protein that can transduce cellular free-energy into biological work by sliding along actin filaments within the cell.
Pneumatic actuation: conversion of compressed air or vacuum energy into mechanical forces resulting in movement.
Strain: in physics, a measure of deformation. It measures the change in configuration relative to the original state when a stress is applied (e.g., during stretching).
Traction force microscopy (TFM): technique to measure the forces that cells exert on their ECM, which can be thought as a proxy for how well focal adhesions are organized and connected to the underlying cytoskeleton.
Uniaxial and/or biaxial stretching: uniaxial, stretching along one axis; biaxial, stretching along two axes; equibiaxial, stretching equally along all axes.

Box 3. Engineering considerations beyond microscopy

When designing a cell-stretching platform, experimental requirements need to be carefully considered and translated into engineering specifications. For example, when stretching is to be combined with other biomechanical characterization methods (e.g., atomic force microscopy to measure cellular stiffness) or specific assays (e.g., infection assays with intracellular pathogens, that often require apical exposure of cells to pathogens and subsequent washes to get rid of extracellularly remaining pathogens), platforms that can provide top access should be considered. Similarly, if the stretching platform is used to mimic a (patho)physiological process (e.g., the effect of intestinal peristalsis on epithelial cell infection or the effect of skin epithelial cell stretching on wound healing), stretching direction(s) (i.e., uniaxial, biaxial, or equibiaxial, see Figure 2A in main text) should also be considered to better represent *in vivo* conditions. Other experimental requirements that would impose platform design restrictions include combination of stretching with other mechanical cues (e.g., fluid flow-induced shear stress imposed on the apex of ECs lining the inner lumen of blood vessels *in vivo*), need for coculture compatible environments (e.g., intestinal epithelium with microbiome, oxic/anoxic interfaces), and need for high-throughput experiments/parallelization (in which case multi-stretcher platforms would be beneficial).

pathways are regulated by stretch-activated channels and can be inhibited by MS [22]. This might be important for developing better drug delivery methods and for combating infection, since many pathogens hijack vesicular-mediated trafficking to infect cells. Additionally, exposure to MS reprograms EC building memory, which is maintained even when cells are placed on non-elastic matrices post-MS [18,23]. Consistent with the notion of mechanical memory, preconditioning of ECs with MS enhances their barrier integrity, a property that is exploited for the creation of well-implantable vascular grafts [24]. However, to better understand tissue and cell (patho) physiology it is paramount to image live cells during exposure to MS and not only after, as commonly done in research laboratories. The literature presented later focuses on CSDs compatible with microscopy. CSDs that enable live-cell imaging during cyclic stretching are also highlighted.

Stretching platforms for live-cell imaging of multicellular assemblies: engineering considerations

Cell-stretching platforms for the study of biological responses of multicellular assemblies to MS have been used in research laboratories for decades. Since the development of the first CSDs in the 1970s [25], a variety of cell-stretching platforms have been developed based on a range of **actuation** principles, including commercially available ones, notably the Flexcell (from Flexcell International) and StrexCell (from STREX Inc.) bioreactors [26]. Those CSDs have been used to answer biological questions of ever-increasing complexity, including how MS influences a variety of cellular functions [27]. Depending on the biological questions posed, several parameters need to be considered in terms of CSD design, capabilities, and automation, including: (i) compatibility with high-resolution live-cell imaging modalities; (ii) compatibility with specific assays (e.g., infection assays that require specific access); (iii) compatibility with (bio)mechanical characterization methods; (iv) biomimetic abilities (e.g., direction(s) of applied stretch); and (v) possibility of simultaneously integrating other physical cues (e.g., shear fluid flow) (Box 3). Later, we specifically focus on CSD compatibility with microscopy, with a special interest in live-cell imaging. In terms of device design, compatibility with live-cell imaging (mainly performed using inverted fluorescence or confocal microscopes) requires a transparent, thin, stretching membrane. Such membranes are fabricated using **elastomeric materials**, most notably polydimethylsiloxane (PDMS) or other silicones, and are actuated either using **motor actuation** or **pneumatic actuation** using vacuum. Other modes of actuation (e.g., piezoelectric, electromagnetic) have been demonstrated but they are not commonly used [28,29].

Motor-actuated cell-stretching platforms

Most laboratory-developed CSDs are actuated using very precise stepper motors (Figure 2B). Cirka *et al*. designed a CSD that utilized stepper motors to apply forces to three corners of a cell culture well [30]. The independently controlled motors allowed for customizable **uniaxial**

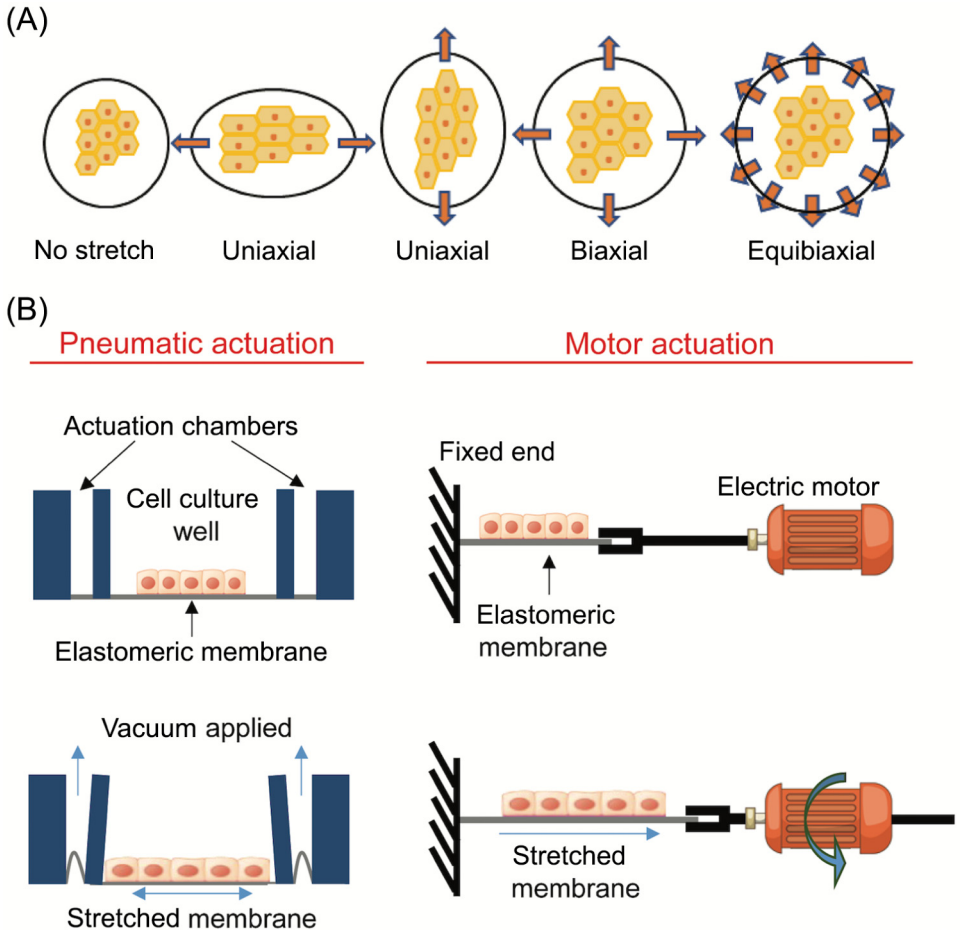

Figure 2. Commonly used actuation principles in cell-stretching devices (CSDs) and modes of stretching. (A) Modes of stretching. From left, uniaxial, biaxial, equibiaxial. This illustration is not meant to show cell alignment upon stretching *in vitro*. (B) Example of pneumatically actuated CSD. In this device, cell culture wells are flanked by (or surrounded by, in the case of circular device architectures) actuation chambers. Elastomeric cell culture membranes are bonded to the devices. When vacuum is applied to the actuation chambers the cell culture membrane is stretched along with the cells cultured on it. (C) Example of a motor-actuated CSD. In this device, an elastomeric membrane is attached to an electric motor through a clamp. The electric motor pulls and stretches the cell culture membrane, exerting strain to the cells attached to it.

and/or biaxial stretching patterns (Figure 2A) on subconfluent interstitial cells of a cardiac valve. Cells cultured in PDMS wells on thin membranes (<150 μm) coated with stiff and soft polyacrylamide hydrogel were stretched for 24 h, after which traction forces exerted by cells on the hydrogel were measured via **traction force microscopy (TFM)**. Imaging was only performed after stretching (in the zero-strain configuration). Due to the flexibility of the membrane, its small thickness, and the large diameter of the cell culture well (22 mm), a custom well holder with a coverslip was necessary to support the membrane during imaging and to prevent the weight of the liquid from causing image distortion. This might ultimately have rendered the device incompatible with live-cell imaging during stretching, since the authors performed TFM at the zero-strain configuration (i.e., after cessation of cyclic stretching). Before developing their own stretching platform, the same group attempted similar investigations using the STREX Cell Stretching System and

Flexcell® Tension System [31]. STREX utilizes a PDMS-based cell culture chamber and a high-precision stepper motor for a consistent range of motion at a variety of speeds and stretch ratios. STREX offers two main CSDs: devices that allow high-throughput long-term stretching and devices that are microscope-mountable for live-cell imaging. However, similar to other PDMS devices, imaging is typically performed before and after stretching and real-time imaging is only possible at low (~10×) magnification [32–34]. Uniaxial and biaxial stretching cannot be combined on a single platform. Flexcell® devices are pneumatically actuated but not compatible with **inverted microscopes**, as flexible silicone membranes are supported by loading posts [26]. Finally, membrane-free devices are also being developed, like the example from Duda *et al*., where a manual CSD was used to strain a *Drosophila melanogaster* wing disc suspended between two PDMS microchannels filled with liquid media. Dynamic stretching was not performed (one stretching cycle per several min and up to 2 h), which made live imaging using a spinning disc confocal microscope possible [35].

Pneumatically actuated cell-stretching platforms

Recently, an example of a pneumatically actuated PDMS-based CSD compatible with high-resolution time-lapse microscopy of adherent cell monolayers was demonstrated by Hart *et al.* [36]. This CSD consisted of a cell culture chamber flanked by two actuation chambers and was sealed using a 125 μm-thick cell culture membrane (Figure 2B). Upon vacuum application, the cell monolayer residing in the cell culture chamber was continuously (as opposed to cyclically) stretched uniaxially at increasing strain and was concurrently observed using transmitted-light, time-lapse microscopy for ~7 h at 1 h intervals. However, optical focus was adjusted manually during stretching, a non-optimal feature, especially if longer/shorter image acquisition intervals are desired. Similar configurations are utilized on organ-on-chip platforms, where suspended elastomeric membranes are pneumatically actuated uniaxially via flanking chambers emulating intestinal peristalsis or respiration [37]. Despite the biomimetic nature of organ-on-chips, imaging capabilities are hindered by the large distance between the microscope objective and the cells, typically exceeding 200 μm. Another imaging-related limitation of devices utilizing suspended membranes arises from the multiple material interfaces separating the cells and the microscope objective (e.g., interfaces between liquid media and elastomers) [38]. In addition to uniaxially stretched cell culture chambers, circular CSDs compatible with high-resolution live-cell imaging have also been demonstrated, utilizing cell culture wells surrounded by actuation ring chambers sealed using thin elastomeric cell culture membranes. When vacuum is applied to the actuation ring chamber, uniform equibiaxial strain is produced across the cell monolayer. Equibiaxial stretching, as opposed to uniaxial discussed earlier, can often better mimic the strain patterns cells experience *in vivo*, depending on the tissue of interest (e.g., IECs during peristalsis). Kreutzer *et al*. reported such a circular stretching platform and used it to induce cardiac differentiation of pluripotent stem cells [39]. Live-cell imaging was performed using an inverted microscope. However, when vacuum was applied to the actuation chamber the cell culture membrane was displaced by ~315 μm in the z-direction. Although an out-of-plane displacement is to be expected upon pneumatic actuation of elastomeric membranes, this limitation is not often discussed in the literature, despite being detrimental for live-cell microscopy. To overcome this issue, Kreutzer *et al*. manually refocused between stretching cycles and used low-magnification objectives to minimize the effect of displacement on image quality [39], similar to what Hart *et al*. reported [36].

Live-cell imaging during cyclic stretching

Although many devices reviewed earlier are compatible with microscopy, live-cell imaging during cyclic stretching has rarely been demonstrated. In the work from Kreutzer, live-cell imaging was performed at long time intervals and focus was manually adjusted between images [39]. In an

earlier work, a similar circular pneumatic CSD was used to apply homogeneous equibiaxial strain to epithelial cells and map cell-matrix stresses before, during, and after stretching [40]. The authors also noted that stretching resulted in lateral cell displacement and defocusing. For TFM and live-cell imaging, manual repositioning/refocusing was performed every 2 min (i.e., after every stretching cycle). In another study, a similar CSD was used to study the mechanisms underlying epithelial fracture during stretching, as this is relevant in many tissues, including the skin [41]. The CSD consisted of a PDMS membrane (80–100 μm thick) clamped between two Teflon rings and placed on top of a circular loading post. Application of vacuum to the outer annular region of the membrane caused uniform equibiaxial strain across the membrane. The cell culture area was large enough to allow top and bottom objective access, making it compatible with inverted and upright optical microscopy. Interestingly, despite the circular pneumatic CSD used, live-cell imaging was demonstrated during cyclic stretching and common problems such as image distortion due to media weight or membrane out-of-plane displacement during stretching were not discussed. Given the relatively slow rate of stretching (10 min stretch pulse performed every 30 min) and the rather short length of experiments (single stretch pulses shown), it is possible that the focus was readjusted after every stretch cycle to correct for membrane deflection. Table 1 summarizes important characteristics of some of the discussed CSDs.

Imaging cells under MS to delve into the crosstalk of cell biochemistry and mechanics

Although technically challenging, attempts have been made to couple exposure of cells to MS *in vitro* with imaging. Such studies lent key insights into how cells function in response to MS, how they transduce biomechanical inputs into signaling, and how this is relevant in biological and biomedical contexts. Later, we outline some seminal studies hoping to encourage further development of CSDs for better coupling with live-cell imaging during stretching.

A study on epithelial cells in monolayer using a pneumatic CSD and videomicroscopy showed that cell migration speeds decrease upon MS but eventually return to baseline levels, suggesting

Table 1. Device examples and their compatibility with microscopy

Mode of stretching	Stretch direction	Microscopy compatibility?	Cyclic stretching?	Strain	Live-cell imaging?	Refs
Pneumatic	Uniaxial	Yes, inverted	No, 7 h, increasing strain every hour	15%	Yes	[36]
Pneumatic	Uniaxial	Yes, inverted, low magnification	Yes, 12 days at 0.2 Hz	15%	No	[37]
Pneumatic	Equibiaxial	Yes, low magnification	Yes, 10 days at increasing rate (0.2–1 Hz)	1-5%	Yes	[39]
Pneumatic	Equibiaxial	Yes, inverted and upright	Yes, 1 s, 1 min, or 10 min pulse every 30 minutes	5%, 10% or 15%	Yes	[41]
Pneumatic	Uniaxial Equibiaxial (different systems)	Yes, upright, immersion	Possible, programmable	Variable	No	[26]
Motor-actuated	Uniaxial and/or biaxial	Yes, inverted	Yes, 24 h at 1 Hz	10%	No	[30]
Screw drive	Uniaxial	Yes, including confocal	No	Up to 100%	Yes	[35]
Motor-actuated	Uniaxial Biaxial (different systems)	Yes, low magnification	Possible, preprogrammed patterns	Based on membrane thickness	Yes	[32–34]

that cells develop homeostatic mechanisms to return to steady state [36]. This was attributed to shuttling of motor protein **myosin II** from the cytoplasm to the cell cortex, while recovery was mediated by an increase in **actin**-myosin contractility, probably due to myosin II being shuttled back into the cytoplasm. Although not explored, alterations in cell–cell and cell–ECM forces, between which there is a high degree of crosstalk, could be the cause of the changes in cell speed [42–44]. Measuring these forces could uncover time-dependent changes in barrier integrity when the epithelium is challenged with MS. Indeed, cell–cell junctions get remodeled in a strain-rate dependent manner and can relax the stress buildup at low strain rates to prevent junction failure [45]. However, when strain magnitude and duration is high, epithelial tissue stretching causes crack formation upon stretch release, the origin of which is not tensile but rather hydraulic [41]. That is, epithelial cracks result from pressure buildup in the ECM during stretch, which supports the hypothesis that epithelial integrity depends on strong coupling between tissue stretching and ECM hydraulics. Using a similar pneumatically actuated CSD, an earlier groundbreaking study (along with further studies that followed) showed that when MS is transiently applied to cells at high strains or rate, cells universally undergo fluidization, reducing their stiffness and disintegrating their cytoskeleton, but at later stages they are able to re-solidify [46–48]. Concurrently, traction stresses exerted by cells to their ECM increase upon acute static MS but drop below baseline levels upon MS release, implying that potential energy is used by cells to reorganize their cytoskeleton and redistribute tension [40]. The magnitude- and strain rate-dependent changes in the mechano-responsiveness of tissues and constituent cells upon MS has also been observed *in vivo* [49]. Whether these time-dependent transitions that cells undergo during MS are sufficient to build persistent mechanical memory remains an active area of research, partly due to its relevance in tissue engineering [8,50].

Imaging coupled with CSDs has accelerated additional fields such as cancer cell and developmental biology. Unlike normal cells, cancer cells typically proliferate and survive better on stiffer ECM, which coincides with loss of ECM rigidity-sensing [51]. Post-MS imaging was used to examine the effect of MS on transformed cancer cell survival from various tissues *in vitro*. The work was motivated by earlier studies on mice with cancer, which were subjected to either MS or exercise and showed a mechanical force-dependent inhibition of tumor growth [52,53]. Using a pneumatically actuated CSD, it was discovered that MS inhibits cancer cell growth and triggers apoptosis ('mechanoptosis'), opposite to what occurs in normal cells. These findings could be exploited further to develop MS-based cancer therapies. Contrary to this study, when epithelial cells in monolayer were mixed with oncogenically transformed cells under stationary conditions, they could get rid of the transformed cells via extrusion. However, when stretched in a pneumatically actuated CSD, transformed cells remained in the monolayer due to changes in their **focal adhesion** dynamics and actomyosin contractility, promoting an invasive cancerous phenotype [54]. The discrepancy between these findings could be explained by the fact that in the latter case cell competition took place in a 'crowded' monolayer and not in subconfluent cells. Imaging during MS could help elucidate how these cell density-dependent alterations emerge. Nonetheless, both studies highlight the importance of considering MS during *in vitro* experiments related to cancer.

MS also contributes to the development of tissues and their mechanisms to cope with mechanical perturbations, as was recently shown by stretching the wing of *Drosophila* while performing live-cell confocal imaging [35]. Upon tissue stretch and at short time scales, myosin II formed asymmetric cables related to actin remodeling. Authors proposed that this is a fast response to stiffen the tissue in an attempt to buffer mechanical perturbations and preserve shape. Future developments could enable examining how variation of MS magnitude and frequencies could impact tissue development. Moreover, in the context of development [55] and during wound

healing [56], MS-sensitive ERK activation traveling in waves from one cell to another faster than diffusion alone, has emerged as a key mechanochemical process that orchestrates cellular motility and tissue organization. Mechanical forces are tightly coupled with cellular signaling, thus directing cell polarization and collective cell migration [56,57]. These studies are based on **Förster resonance energy transfer (FRET)**-based biosensors designed to follow ERK activation over time and space using microscopy modalities. Combining such measurements with biomechanics techniques (e.g., TFM) unambiguously proved the importance of interrogating the crosstalk between mechanics and biochemical signaling to understand how tissues are shaped and how they return to homeostasis upon insults (e.g., a wound). Since ERK is activated by MS, future developments could enable determining the role of cyclic MS, as it occurs in so many tissues and partakes in regulating the dynamics of signaling events across multicellular assemblies, such as those seen upon ERK activation in stationary cultures.

Concluding remarks and future perspectives

What is holding current CSDs back from broad application in live-cell imaging during cyclic stretching (see Outstanding questions)? Limitations include elastomeric membrane displacement during stretching, moving cells away from the focal plane imaged, thus affecting image quality; device incompatibility with inverted microscopy due to the need for supporting surfaces; and long distances between suspended membranes and microscope objectives. Depending on the experimental requirements, workarounds have been proposed, such as manually adjusting the optical focus during stretching (only feasible when stretching rate is low, stretch pulses are long, and no long-term time-lapse imaging is needed) and using low magnification objectives. Beyond these limitations, live-cell imaging during MS introduces further engineering requirements, especially when performed over long periods. For example, the effects of microscope focal drift become more dramatic when combined with a dynamically stretching system. In such cases, a robust z-drift compensator might be necessary. Membrane displacement could also be compensated by robust microscope autofocus systems, especially when manual refocusing is not feasible or practical. Spinning disk confocal microscopes might be the preferred option for live-cell imaging on CSDs since they offer improved temporal resolution and decrease the risk of photobleaching and phototoxicity compared with laser scanning confocal microscopes. Compared with traditional epifluorescence microscopes, they offer enhanced lateral and axial resolution, decrease the risk of out of focus light, or aberrations in the case of thick samples (as when cells are seeded on a PDMS stretchable membrane), as well as of bleed-through and blurring. In terms of materials, beyond determining membrane thickness and level of transparency, characterizing changes in membrane properties during cyclic stretching is also necessary. Ideally, the membrane should exhibit low **hysteresis**, high toughness (resistance to rupture), and fatigue resistance (prolonged survival under cyclic loads). However, most existing stretchable materials cannot meet all three requirements simultaneously, although promising novel composites have been recently reported [58]. Finally, for time-lapse imaging, and depending on the complexity and frequency of stretching patterns, automation requirements should be considered.

Given that (extra)cellular physical forces, such as stretching forces, play a determinant role in shaping biological processes from cell migration to tissue morphogenesis, it is of utmost importance to develop platforms that enable examination of cell- and tissue-level behaviors in response to MS. That would allow a better understanding of how forces can act as a cue to preserve tissue integrity and maintain tissue homeostasis in space and time. It would also help elucidate how cells convert extrinsic mechanical forces into intrinsic biochemical signals, thus eliciting specific cellular responses. In the future, such knowledge could be used to direct cellular behavior to combat disease (e.g., cancer) or to develop better constructs for tissue engineering.

Outstanding questions

A large variety of biological and biomedical questions could be answered using cell-stretching platforms coupled with concurrent or subsequent live-cell imaging. Their application is, however, limited, despite some commercial options. What is holding current CSD back from broad application? How could CSDs be built so to also enable high-throughput experimentation and compatibility with videomicroscopy?

Most live-cell microscopy imaging during cyclic stretching is performed using low stretch rates and long intervals between image acquisitions of cells residing on 2D matrices. Could we better understand the processes behind various diseases associated with defects in mechanotransduction, by developing better cell-stretching platforms that allow for live-cell imaging during cyclic stretching at high rates on both 2D and 3D matrices?

Which limitations related to live-cell imaging during stretching could be decoupled from the stretching device used and be compensated by specialized microscope equipment? What would an ideal microscope include for live-cell imaging during stretching? Would such a system allow performing FRET imaging or other types of more sophisticated imaging modalities to extract information on the dynamics of biochemical cell signaling processes during MS application?

Could one concurrently measure the traction forces exerted by cells on their ECM (e.g., using TFM) as well as on neighboring cells in monolayer, which can be thought as a proxy for barrier integrity? Could one further reveal how cellular biomechanics crosstalk with biochemical signaling during MS, thus accelerating the field of mechanobiology?

Most elastomeric membranes used in stretching experiments are silicon-based. Could stretching experiments benefit from nonsilicone-based membranes? Are there promising candidate materials that would also facilitate long-term live-cell imaging? Could such developments benefit the field of tissue engineering by further increasing

the mechanical strength and functioning of engineered tissue to eventually ameliorate successful implantation into patients?

Acknowledgments

This research was supported by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy – EXC 2124 – 390838134 (E.E.B.), by the DFG grant, Project number 490839690 (E.E.B, I.C.) and by the SMART BIOTECS alliance between the Technische Universität Braunschweig and the Leibniz Universität Hannover (I.C.). This initiative is supported by the Ministry of Science and Culture (MWK) of Lower Saxony, Germany. We thank Libera Lo Presti for discussions and for revising the manuscript and David Jaworksi for originally designing part of Figure 2. Figure I in Box 2 was created in part with BioRender.com.

Declarations of interest

No interests are declared.

References

1. Janmey, P.A. *et al.* (2019) Stiffness sensing by cells. *Physiol. Rev.* 100, 695–724
2. Vining, K.H. and Mooney, D.J. (2017) Mechanical forces direct stem cell behaviour in development and regeneration. *Nat. Rev. Mol. Cell Biol.* 18, 728–742
3. Jufri, N.F. *et al.* (2015) Mechanical stretch: physiological and pathological implications for human vascular endothelial cells. *Vasc. Cell* 7, 8
4. Shradhanjali, A. *et al.* (2015) Cardiomyocyte stretching for regenerative medicine and hypertrophy study. *Tissue Eng. Regen. Med.* 12, 398–409
5. Engler, A.J. *et al.* (2006) Matrix elasticity directs stem cell lineage specification. *Cell* 126, 677–689
6. Trepat, X. *et al.* (2009) Physical forces during collective cell migration. *Nat. Phys.* 5, 426–430
7. Riehl, B.D. *et al.* (2012) Mechanical stretching for tissue engineering: two-dimensional and three-dimensional constructs. *Tissue Eng. Part B Rev.* 18, 288–300
8. Mihic, A. *et al.* (2014) The effect of cyclic stretch on maturation and 3D tissue formation of human embryonic stem cell-derived cardiomyocytes. *Biomaterials* 35, 2798–2808
9. Wong, N.R. *et al.* (2021) Resident cardiac macrophages mediate adaptive myocardial remodeling. *Immunity* 54, 2072–2088
10. Kasendra, M. *et al.* (2020) Duodenum intestine-chip for preclinical drug assessment in a human relevant model. *eLife* 9, e50135
11. Bai, L. *et al.* (2019) Genetic identification of vagal sensory neurons that control feeding. *Cell* 179, 1129–1143
12. Kim, H.J. *et al.* (2012) Human gut-on-a-chip inhabited by microbial flora that experiences intestinal peristalsis-like motions and flow. *Lab Chip* 12, 2165–2174
13. Kim, H.J. *et al.* (2016) Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. *Proc. Natl. Acad. Sci. U. S. A.* 113, E7–E15
14. Ghoshal, U.C. and Ghoshal, U. (2017) Small intestinal bacterial overgrowth and other intestinal disorders. *Gastroenterol. Clin.* 46, 103–120
15. Park, J.-A. *et al.* (2015) Putting the squeeze on airway epithelia. *Physiology (Bethesda)* 30, 293–303
16. Yi, E. *et al.* (2016) Mechanical forces accelerate collagen digestion by bacterial collagenase in lung tissue strips. *Front. Physiol.* 7, 287
17. Russo, T.A. *et al.* (2018) Mechanical stretch implications for vascular endothelial cells: altered extracellular matrix synthesis and remodeling in pathological conditions. *Life Sci.* 213, 214–225
18. Birukov, K.G. *et al.* (2003) Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch. *Am. J. Physiol. Lung Cell Mol. Physiol.* 285, L785–L797
19. Gawlak, G. *et al.* (2016) Chronic high-magnitude cyclic stretch stimulates EC inflammatory response via VEGF receptor 2-dependent mechanism. *Am. J. Physiol. Lung Cell Mol. Physiol.* 310, L1062–L1070
20. Gervásio, O.L. *et al.* (2011) Caveolae respond to cell stretch and contribute to stretch-induced signaling. *J. Cell Sci.* 124, 3581–3590
21. Thottacherry, J.J. *et al.* (2018) Mechanochemical feedback control of dynamin independent endocytosis modulates membrane tension in adherent cells. *Nat. Commun.* 9, 4217
22. Skokan, T.D. *et al.* (2021) Mechanical stretch regulates macropinocytosis in *Hydra vulgaris*. *bioRxiv* Published online December 4, 2021. https://doi.org/10.1101/2021.12.03.471193
23. Birukova, A.A. *et al.* (2008) Long-term cyclic stretch controls pulmonary endothelial permeability at translational and post-translational levels. *Exp. Cell Res.* 314, 3466–3477
24. Luo, J. *et al.* (2020) Tissue-engineered vascular grafts with advanced mechanical strength from human iPSCs. *Cell Stem Cell* 26, 251–261
25. Leung, D.Y.M. *et al.* (1977) A new in vitro system for studying cell response to mechanical stimulation: different effects of cyclic stretching and agitation on smooth muscle cell biosynthesis. *Exp. Cell Res.* 109, 285–298
26. Bartalena, G. *et al.* (2011) A novel method for assessing adherent single-cell stiffness in tension: design and testing of a substrate-based live cell functional imaging device. *Biomed. Microdevices* 13, 291–301
27. Mammoto, A. *et al.* (2012) Mechanosensitive mechanisms in transcriptional regulation. *J. Cell Sci.* 125, 3061–3073
28. Kamotani, Y. *et al.* (2008) Individually programmable cell stretching microwell arrays actuated by a Braille display. *Biomaterials* 29, 2646–2655
29. Kamble, H. *et al.* (2016) Cell stretching devices as research tools: engineering and biological considerations. *Lab Chip* 16, 3193–3203
30. Cirka, H. *et al.* (2016) Active traction force response to long-term cyclic stretch is dependent on cell pre-stress. *Biophys. J.* 110, 1845–1857
31. Throm Quinlan, A.M. *et al.* (2011) Combining dynamic stretch and tunable stiffness to probe cell mechanobiology in vitro. *PLoS One* 6, e23272
32. Akiyama, Y. *et al.* (2018) Poly(N-isopropylacrylamide)-grafted polydimethylsiloxane substrate for controlling cell adhesion and detachment by dual stimulation of temperature and mechanical stress. *Biomacromolecules* 19, 4014–4022
33. Yuge, S. *et al.* (2022) Mechanical loading of intraluminal pressure mediates wound angiogenesis by regulating the TOCA family of F-BAR proteins. *Nat. Commun.* 13, 2594
34. Tsujita, K. *et al.* (2021) Homeostatic membrane tension constrains cancer cell dissemination by counteracting BAR protein assembly. *Nat. Commun.* 12, 5930
35. Duda, M. *et al.* (2019) Polarization of myosin II refines tissue material properties to buffer mechanical stress. *Dev. Cell* 48, 245–260
36. Hart, K.C. *et al.* (2021) An easy-to-fabricate cell stretcher reveals density-dependent mechanical regulation of collective cell movements in epithelia. *Cell. Mol. Bioeng.* 14, 569–581
37. Kasendra, M. *et al.* (2018) Development of a primary human small intestine-on-a-chip using biopsy-derived organoids. *Sci. Rep.* 8, 2871
38. Sim, J.Y. *et al.* (2012) Uniaxial cell stretcher enables high resolution live cell imaging. In *2012 IEEE 25th International Conference on Micro Electro Mechanical Systems (MEMS)*, pp. 854–857, IEEE
39. Kreutzer, J. *et al.* (2014) Pneumatic cell stretching system for cardiac differentiation and culture. *Med. Eng. Phys.* 36, 496–501
40. Gavara, N. *et al.* (2008) Mapping cell-matrix stresses during stretch reveals inelastic reorganization of the cytoskeleton. *Biophys. J.* 95, 464–471

41. Casares, L. *et al.* (2015) Hydraulic fracture during epithelial stretching. *Nat. Mater.* 14, 343–351
42. Aparicio-Yuste, R. *et al.* (2022) A stiff extracellular matrix favors the mechanical cell competition that leads to extrusion of bacterially-infected epithelial cells. *Front. Cell Dev. Biol.* 10, 912318
43. Bazellières, E. *et al.* (2015) Control of cell–cell forces and collective cell dynamics by the intercellular adhesome. *Nat. Cell Biol.* 17, 409–420
44. Yuste, R.A. *et al.* (2022) *Borrelia burgdorferi* modulates the physical forces and immunity signaling in endothelial cells. *iScience* 25, 104793
45. Esfahani, A.M. *et al.* (2021) Characterization of the strain-rate-dependent mechanical response of single cell-cell junctions. *Proc. Natl. Acad. Sci. U. S. A.* 118, e2019347118
46. Trepat, X. *et al.* (2007) Universal physical responses to stretch in the living cell. *Nature* 447, 592–595
47. Nava, M.M. *et al.* (2020) Heterochromatin-driven nuclear softening protects the genome against mechanical stress-induced damage. *Cell* 181, 800–817
48. Chen, C. *et al.* (2010) Fluidization and resolidification of the human bladder smooth muscle cell in response to transient stretch. *PLoS One* 5, e12035
49. Andreu, I. *et al.* (2021) The force loading rate drives cell mechanosensing through both reinforcement and cytoskeletal softening. *Nat. Commun.* 12, 4229
50. Stickler, P. *et al.* (2010) Cyclically stretching developing tissue in vivo enhances mechanical strength and organization of vascular grafts. *Acta Biomater.* 6, 2448–2456
51. Tijore, A. *et al.* (2021) Selective killing of transformed cells by mechanical stretch. *Biomaterials* 275, 120866
52. Berrueta, L. *et al.* (2018) Stretching reduces tumor growth in a mouse breast cancer model. *Sci. Rep.* 8, 7864
53. Betof, A.S. *et al.* (2015) Modulation of murine breast tumor vascularity, hypoxia and chemotherapeutic response by exercise. *J. Natl. Cancer Inst.* 107, djv040
54. Chagnon-Lessard, S. *et al.* (2021) Mechanotransduction of strain regulates an invasive phenotype in newly transformed epithelial cells. *Front. Phys.* 9, 654613
55. Ishii, M. *et al.* (2021) Retrograde ERK activation waves drive base-to-apex multicellular flow in murine cochlear duct morphogenesis. *eLife* 10, e61092
56. Hino, N. *et al.* (2020) ERK-mediated mechanochemical waves direct collective cell polarization. *Dev. Cell* 53, 646–660
57. Hino, N. *et al.* (2022) A feedback loop between lamellipodial extension and HGF-ERK signaling specifies leader cells during collective cell migration. *Dev. Cell* 57, 2290–2304
58. Wang, Z. *et al.* (2019) Stretchable materials of high toughness and low hysteresis. *Proc. Natl. Acad. Sci. U. S. A.* 116, 5967–5972
59. Fletcher, D.A. and Mullins, R.D. (2010) Cell mechanics and the cytoskeleton. *Nature* 463, 485–492
60. Schwarz, U.S. *et al.* (2006) Focal adhesions as mechanosensors: the two-spring model. *Biosystems* 83, 225–232
61. Bastounis, E. *et al.* (2014) Both contractile axial and lateral traction force dynamics drive amoeboid cell motility. *J. Cell Biol.* 204, 1045–1061
62. Alvarez-Gonzalez, B. *et al.* (2015) Three-dimensional balance of cortical tension and axial contractility enables fast amoeboid migration. *Biophys. J.* 108, 821–832
63. Choquet, D. *et al.* (1997) Extracellular matrix rigidity causes strengthening of integrin- cytoskeleton linkages. *Cell* 88, 39–48
64. Bastounis, E.E. *et al.* (2021) Mechanical competition triggered by innate immune signaling drives the collective extrusion of bacterially infected epithelial cells. *Dev. Cell* 56, 443–460
65. Kudoh, S. *et al.* (1998) Mechanical stretch induces hypertrophic responses in cardiac myocytes of angiotensin II type 1a receptor knockout mice. *J. Biol. Chem.* 273, 24037–24043
66. Santus, P. *et al.* (2019) The airways' mechanical stress in lung disease: implications for COPD pathophysiology and treatment evaluation. *Can. Respir. J.* 2019, 3546056
67. Bastounis, E.E. *et al.* (2022) Mechanical forces govern interactions of host cells with intracellular bacterial pathogens. *Microbiol. Mol. Biol. Rev.* e0009420
68. Kılıç, A. *et al.* (2020) Mechanical forces induce an asthma gene signature in healthy airway epithelial cells. *Sci. Rep.* 10, 966
69. Neto, F. *et al.* (2018) YAP and TAZ regulate adherens junction dynamics and endothelial cell distribution during vascular development. *eLife* 7, e31037
70. Gudipaty, S.A. *et al.* (2017) Mechanical stretch triggers rapid epithelial cell division through Piezo1. *Nature* 543, 118–121
71. Wang, Y. *et al.* (2020) Multiple effects of mechanical stretch on myogenic progenitor cells. *Stem Cells Dev.* 29, 336–352