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TOPICAL REVIEW

# Cell stretching devices integrated with live cell imaging: a powerful approach to study how cells react to mechanical cues

**Beatrice Bighi**[1,2], **Gregorio Ragazzini**[3], **Alessia Gallerani**[1], **Andrea Mescola**[2], **Chiara Scagliarini**[1], **Chiara Zannini**[3,4], **Martina Marcuzzi**[5], **Elena Olivi**[3], **Claudia Cavallini**[3,4], **Riccardo Tassinari**[3], **Michele Bianchi**[6], **Lorenzo Corsi**[6], **Carlo Ventura**[3,4] **and Andrea Alessandrini**[1,2,*]

[1] Department of Physics, Informatics and Mathematics, University of Modena and Reggio Emilia, via Campi 213/A, 41125 Modena, Italy
[2] CNR-Nanoscience Institute-S3, via Campi 213/A, 41125 Modena, Italy
[3] Eldor Lab, via di Corticella 183, 40128 Bologna, Italy
[4] National Laboratory of Molecular Biology and Stem Cell Engineering, National Institute of Biostructures and Biosystems (I.N.B.B.), via di Corticella 183, 40128 Bologna, Italy
[5] Department of Medical and Surgical Sciences, University of Bologna, via G. Massarenti 9, Bologna 40138, Italy
[6] Department of Life Sciences, University of Modena and Reggio Emilia, Via G. Campi 287, 41125 Modena, Italy
* Author to whom any correspondence should be addressed.

**E-mail:** andrea.alessandrini@unimore.it

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## Abstract

Mechanical stimuli have multiple effects on cell behavior, affecting a number of cellular processes including orientation, proliferation or apoptosis, migration and invasion, the production of extracellular matrix proteins, the activation and translocation of transcription factors, the expression of different genes such as those involved in inflammation and the reprogramming of cell fate. The recent development of cell stretching devices has paved the way for the study of cell reactions to stretching stimuli *in-vitro*, reproducing physiological situations that are experienced by cells in many tissues and related to functions such as breathing, heart beating and digestion. In this work, we review the highly-relevant contributions cell stretching devices can provide in the field of mechanobiology. We then provide the details for the in-house construction and operation of these devices, starting from the systems that we already developed and tested. We also review some examples where cell stretchers can supply meaningful insights into mechanobiology topics and we introduce new results from our exploitation of these devices.

## Glossary

| | |
|---|---|
| Mechanosensing | All the mechanisms by which cells can sense mechanical forces from the external environment |
| Mechanotransduction | All the processes by which cells, after receiving a mechanical stimulus, convert it into a biochemical signal |
| Uniaxial | Type of deformation occurring by actively stretching a substrate along one axis |
| Biaxial | Type of deformation occurring by actively stretching a substrate along two perpendicular axis |
| Isotropic | Type of deformation occurring in equal entity along all the possible in plane directions |
| Pneumatic actuation | Type of actuation occurring by changing the pressure (increasing or decreasing) in a closed chamber in contact with the cell substrate |
| Motor actuation | Type of actuation obtained by exploiting typically an electrical signal to drive a motor producing a stretching of the substrate |
| Strain | Percentage change of a geometrical configuration with respect to the initial one |

| | |
|---|---|
| Minimum/zero strain direction | Direction along which the strain of a substrate is minimum or zero. It can be zero if the strains along two perpendicular directions are opposite |
| Extracellular matrix (ECM) | Network of proteins and other molecules surrounding cells. This matrix act as active physical support for cell adhesion, spreading and migration, allowing cells to communicate with other cells as well as the diffusion of molecules, including messengers and metabolites |
| Stress fibers | Higher order cytoskeletal structures obtained by the cross-linking of several actin filament bundles. Together with myosin motor proteins they produce cell contraction |
| Molecular-Clutch mechanism | Analytical description of the force transmitted from a cell to the substrate based on the activity of myosin and stress fibers and on/off rates of chemical bonds |
| Catch-bonds | Type of bond between two molecular partners in which the application of a pulling force produces a stabilization of the bond itself |
| Slip bonds | Bonds whose lifetime monotonically decreases when the mechanical force applied to them increases |
| Focal adhesion complexes | Large macromolecular complexes that establish cell adhesion to the ECM. They transmit mechanical forces from the inside to the outside of the cell and vice-versa by establishing a bridge between the ECM and the cell cytoskeleton |
| LINC proteins | Complex of molecules spanning the nuclear membranes (Linker of Nucleoskeleton and Cytoskeleton) that transmits cytoskeletal forces to the nuclear surface |

## 1. Introduction

Since the introduction of cell culturing techniques at the beginning of the twentieth century by Harrison [1, 2], many biochemical assays have been performed on *in-vitro* cells. These assays generally aim to study how cells react, in terms of gene expression or phenotypic properties, to different biochemical or physical stimuli. The same cell cultures were also established as models of different diseases. Accordingly, besides being the smallest units of life, cells were also considered as the fundamental units of pathological processes. The cell culturing substrates of the first attempts, and for a long time to follow, were based on glass. Afterwards, the glass substrates, when not necessarily requested to improve imaging methods, as in the case of fluorescence microscopy, were replaced by polystyrene surfaces. To date, the vast majority of the disposables for cell culturing are made in polystyrene, being a low-cost and easy-to-handle material. These surfaces, when used for culturing adherent cells, are always adequately conditioned with proteins of the extracellular matrix (ECM) to enhance cell adhesion. About fifty years ago, it became clear that the physical and chemical properties of the adhesion surface could affect cell behavior in terms of morphology, cytoskeleton organization, migration, differentiation and gene expression [3–5]. In particular, many investigations established that cell behavior is strongly dependent on the mechanical properties of the substrate on which they are seeded [6–8] and the mechanobiology at the level of single cells is closely related to the mechanics of the tissues to which the cells originally belong. Therefore, many studies concentrated on different culturing conditions, exploiting materials whose rheological properties could be properly tuned to reproduce conditions found in physiological environments. To better reproduce stimuli due to cell-cell and cell-ECM interactions, a breakthrough in cell culturing approaches was represented by the introduction of 3D microenvironments for *in-vitro* cells [9, 10]. By these approaches, it is possible to better recapitulate *in-vivo* conditions to efficiently predict the response of cells to different stimuli, such as drugs. At the same time, 2D cell migration for many aspects is not representative of cell migration in a 3D environment, where steric obstacles have to be faced and overcome by cells [11–13]. Moving in the direction of an even better reproduction of *in-vivo* conditions for living cells, it cannot be overlooked that many cells in the body reside in tissues that are continuously exposed to mechanical forces and, in some cases, the forces experienced have a cyclical character. In order to implement this possibility for cell culturing systems, devices in which cells are cultured on stretchable membranes and are subjected to stretching protocols simulating physiological processes such as digestion, breathing or the pulsating behavior of the vascular system, have been developed [14–18]. These approaches work for both 2D and 3D culture models [19–21]. It has been shown, even if in some specific cases with contrasting results, that a stretching protocol is able to affect gene expression, cell differentiation, migration, proliferation, morphology, cell alignment and the secretion of proteins of the ECM that, on their turn, affect the cell environment and consequently also the behavior of the same cells, establishing in specific cases a sort of positive feedback. Forces applied by cells on their environment are in many cases equivalent, in terms of cell reactions, to forces applied on cells from the ECM [22] (see section 2). From this point of view, studies of cell reactions to forces transmitted by the substrates, as it happens in cell stretching devices, appear extremely relevant in the context of mechanobiology similarly to active forces produced by cells.

Some commercial devices that can be exploited to deliver specific stretching protocols to cells are already on the market (see table 1 for a list of some models already on the market), but many reports in the literature

are based on custom-developed apparatus [23–26]. As for the traditional cell culturing conditions, the possibility of live-imaging while stretching cells, strongly enhances the understanding of how mechanical cues can affect cell behavior, for example providing the kinetics of the homeostatic rearrangement of cells after a change of the mechanical signal exerted on them. Accordingly, devices with optical access and with the option to position them on top of an optical microscope stage have been developed [27–30]. Cell stretchers with optical access and live-cell imaging enable the observation of the kinetics of cell adaptation to mechanical stimuli with high time resolution and could favor the validation of already developed quantitative analytical theory for these processes, as in the case of cell orientation (see section 5.1).

In this work, we will focus on the use of stretcher devices to investigate specific aspects of mechanobiology, concentrating also on the technical aspects of the different devices that we developed and tested and which allow the application of custom stretching protocols to cells according to various symmetries, while acquiring time-lapse sequences of the cells by optical microscopy. The work is organized as follows. In section 2 we will introduce a general analysis of what cell stretchers can do in the context of mechanobiology, focusing on the molecular mechanism of force sensing and transduction. We will give particular emphasis on the roles of different applied frequencies or strain/loading rate, and of the amplitude of the strain. In section 3 we will present an overview of the various strain actuation strategies that can be found in the literature and the specific construction details we adopted for the devices we implemented. In many cases, to fully understand how cells respond to stimuli in terms of proliferation, migration and orientation, it is important to have a complete strain map of the surface on which cells are growing. This will allow to obtain a map of the directions of maximum, minimum or zero strain and of the strain gradient. For example, in many cases, the comparison of the experimental results regarding cell orientation upon a directional stretching stimulus with analytical theories requires an optimal control over the strain map of the elastomeric substrate. Accordingly, in section 4 we will explain the theory of the strain measurements and the procedures to obtain a correct calibration of the strain map on the surface of the elastomers on which cells are seeded. This analysis will be complemented by a comparison between experimental strain calibrations and finite element methods (FEMs) simulations. Section 5 will deal with some examples of the application of these devices in cell biology from our and other groups, concentrating on the contribution of this approach in the elucidation of the cellular bases of some diseases. In section 6 we will consider the possibility of integrating stretching devices with traction force microscopy (TFM) to reveal the stress applied by cells on the surface when they are exposed to stretching stimuli. At the end, in section 7, possible developments of these devices for both increasing the understanding of molecular aspects of mechanobiology and for their exploitation for the preconditioning of tissues to be used in regenerative medicine will be considered. We will also consider the current limitations of these techniques. We believe this work could be of inspiration for extending the studies of the role of mechanical stimuli on cells, especially considering the specificity of the signal, in terms of frequency, amplitude and duration. We speculate that the cells in the different tissues could work as band-pass filters for different mechanical cues and that the dynamical aspect of these signals could be relevant for development of tissues and for their regeneration.

## 2. Mechanobiology with cell stretching devices

Cell stretching devices can be exploited to investigate different aspects of cellular mechanobiology. In particular, stretching devices allow investigating the effects of forces applied from outside (figure 1(a)) on cell behavior by providing specific force stimuli with controlled amplitude, frequency and directionality. This is particularly relevant in the context of cells belonging to tissues that are physiologically subjected to cyclic stretching, such as cells of the heart and of the blood vessels during heart beating, cells of the lung during breathing and cells in the intestine during peristalsis.

Mechanobiology focuses on the relevance of forces in the behavior of biological systems, and here we have focused specifically on living cells. In this context, mechanobiology refers to both forces applied by cells to their environment (forces from inside) and forces applied by the environment to cells (forces from outside). In the first case, the acto-myosin complex is primarily responsible for the application of force. Thanks to the myosin cycle, which utilizes ATP, traction forces are applied to stress fibers and transmitted to the external environment via molecular bridges that include integrins, adaptor proteins such as talin and vinculin, and molecules from the ECM. It has been found that the force transmitted from the inside of the cell to the external environment depends on the mechanical properties of the environment itself [31]. In particular, starting with a very soft ECM, the force transmitted to the environment, which can be measured by TFM [32], increases with a stiffer environment [33–36]. Experimentally, it has been found that in some cases the transmitted force increases continuously as the stiffness of the support increases [32, 35, 36], whereas in other cases the transmitted force has a biphasic behavior as the stiffness of the environment

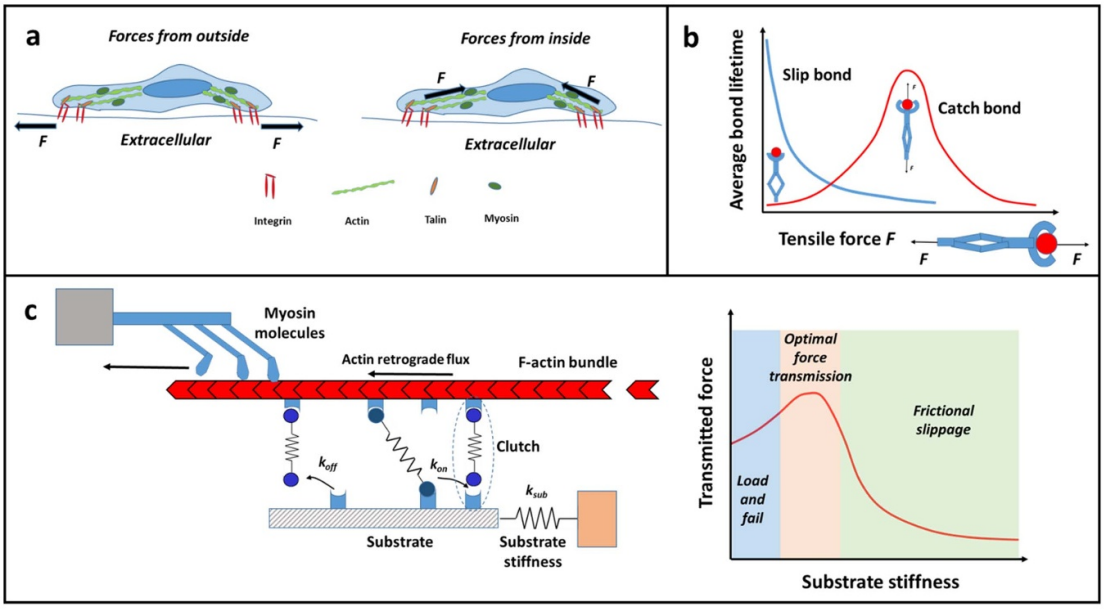


**Figure 1.** (a) Forces from outside applied on cells by the ECM and forces from inside applied by the myosin/actin complex in the cell. The two situations can be described equivalently by a similar molecular clutch model.; (b) difference between a slip-bond, for which the average bond lifetime decreases upon an increase of the disjoining applied force (following a Bell behavior described by one exponential) and a catch-bond (or catch-slip-bond) for which the lifetime initially increases upon the application of a pulling force followed by a regime in which the detachment is again faster for higher forces. This type of bond can be modeled using two exponential functions. (c) Basic scheme of the molecular clutch model. Myosin motors apply a traction force on the actin bundles and the force is transmitted to the substrate via integrin and other adaptor proteins. An initial substrate rigidity increase produces a rise of the loading force rate and, consequently, of the force at which ligands disengage, and the transmitted force increases. In fact, the substrate rigidity affects the rate of force increase and the unbinding force of molecular bonds depends on this rate. For a very soft substrate the force transmitted to the substrate rises very slowly and molecular bonds have enough time to engage, but, once the force gets to a high value (critical force), the rupture of a bond induces the disengage of all the bonds and the process starts again (load-and-fail regime). Increasing the stiffness of the substrate, a higher transmitted critical force can be reached. For very rigid substrates, high values of force are reached very rapidly (before the number of bonds can sufficiently increase) inducing disengagement of the molecular bonds and producing a low value of transmitted force (frictional slippage). A qualitative plot of the transmitted force as a function of substrate stiffness is also reported below the scheme.

increases [37]. The biphasic behavior has been reproduced by an analytical model initially developed by Mitchison and Kirschner [38] and then translated into a computationally available model by Chan and Odde [33]. The model is based on the traction force produced by myosin-actin bundles and transmitted by molecular structures such as talin, integrins, focal adhesion complexes and proteins of the ECM (the 'clutches', which can be described as springs) to the substrate on which the cells grow. In turn, the rigidity of the substrate is characterized by an elastic constant. The model intrinsically depends on the rate of force increase produced by the myosin-actin system, which causes a retrograde flow of actin from the outer to the inner region of the cell. In fact, the clutches are characterized by binding and unbinding rates and the unbinding rate depends on the force applied according to the Bell model [39, 40]. The relevant unbinding rate is typically that of the weakest bond which, in several cases, can be assumed to be the integrin/ECM proteins bond, although other molecules involved can be important [41]. At the same time, the binding rate is affected by the density of integrin molecules (in some cases also CD44 proteins [42]) in the cell membrane and also of the ligands. The retrograde flow of actin due to myosin motors is described by a linear force-velocity relationship as in the case of muscles. The rate of the actomyosin cycle influences the retrograde velocity and, consequently, the rate of force loading, which can be expressed as the product of the elastic constant of the substrate and the speed of the retrograde flow. Starting from disengaged ligands, bonds begin to form between the cell and the substrate, and the force transmitted increases as new bonds are formed (figure 1(c)). At a certain value of the force, a collapse of all the established bonds occurs, the transmitted force goes to zero and the entire process starts again (load-and-fail regime). When the stiffness of the substrate increases, the force loading rate is affected. If the loading rate of the bonds increases, the bonds are able to reach a higher value of rupture/breaking force and the transmitted force increases. For a certain critical value of the stiffness, the force loading rate on the ligands is so high that the unbinding rate is higher than the binding one and the transmitted force decreases (frictional slippage). It is important to emphasize that many of the parameters describing the clutch model are derived from experiments on single molecules. Cells and their environment have many possibilities to control these parameters and to act on the behavior of

cell (e.g. number of motors, density of integrins, rates of binding/unbinding) and, according to the molecular clutch model, it appears that the main parameter controlling the behavior of cells is the rate of force increase along the mechanical chain connecting the cell nucleus to the environment. This process explains the sensitivity of cells to substrate rigidity and also the relevance of the signal frequency in the application of forces. Activation of mechanosensing pathways in many cases occurs when cells are able to exert a high traction force on the environment. The initial model by Chan and Odde predicted a biphasic behavior of the cell traction force as a function of substrate stiffness, but, in several cases, a monotonic and continuously increasing traction force is observed upon an increase of the matrix rigidity. This behavior can be explained by considering the presence of adaptor proteins such as talin and their partial denaturation upon force increase before the clutch disengages, followed by the binding of vinculin, which reinforces the focal adhesion complexes [32, 35, 36, 43–45], and produces their complete maturation. Talin unfolding obeys a slip bond behavior (figure 1(b)) whereas, other molecular partners such as integrin and fibronectin obey a catch-bond model. In the molecular chain, depending on the level of force obtained, one or the other element of the chain will be the weakest.

The molecular clutch model has been developed for the active force applied by a cell on its environment. Andreu *et al* [29, 46] demonstrated that, by taking cells on a soft substrate, for which mechanosensory events like Yes Associated Protein (YAP) translocation inside the nucleus or extension of focal adhesion complexes are not induced, the rate of force application by means of a cyclic stretcher can be used as a control parameter to induce mechanosensing events, as if the cell was growing on a stiffer support. Previous work by Cui *et al* had already demonstrated that cells growing on a soft substrate, typically characterized by a small spreading area, when exposed to cyclic stretching of the substrate, adopt the typical configuration of cells growing on a hard support, where they typically have a large spreading area, forming enhanced stress fibers and large regions of focal adhesion [20, 22]. The interesting aspect of Andreu *et al* work is that, by introducing very small changes to the molecular clutch model, such as the force now increasing due to the stretching process from outside and the possibility, for very high force loading rates, besides integrin disengagement, of actin fluidization leading to the unbinding of the clutch, they could reproduce the experimental findings. In particular, they reproduced the biphasic trend of the cited events of mechanosensing upon an increase of the loading rate. The decrease of YAP translocation inside the nucleus for high loading rates is now interpreted as a fluidization event in which a major reorganization of the actin cytoskeleton occurs and the force from outside is no longer transmitted to the nucleus. This analysis suggests that the molecular determinants ruling the behavior of cells when force is internally produced by the actomyosin system or when the force is applied from outside by stretching, are very similar. This aspect reinforces the relevance of experiments performed by cyclic stretching devices offering the possibility of exploiting already developed biochemical models. The force increase experienced in the classical clutch model by the action of the actin-myosin system and the corresponding retrograde flow speed [47], are in this case related to the loading rate of the stretcher, but the possibility of actin fibers softening by a fluidization mechanism is introduced [48]. The fluidization is due to molecular events that control the organization of the cytoskeleton producing a more viscous-like behavior of the cell (hence the name of the process). In the works by Cui *et al* and Andreu *et al* [22, 46] it has been shown that the fluidization of cells, measured also by atomic force microscopy, is controlled by the force increasing rate and higher values of this parameter produce actin disruption before the disengagement of integrin molecules and talin unfolding. In this case it is assumed that the specific direction of pulling, from-outside-to-inside or from-inside-to-outside, is not relevant in the model. However, it has been demonstrated that the presence of catch-bonds in the adaptors complex, in particular the vinculin-actin bond, is sensitive to the specific direction of the force. Specifically, the catch-bond character is particularly strong only if forces are applied towards the minus end of actin [49]. In this sense, active forces by cells could produce subtle different effects with respect to forces of equal amplitude but applied by a stretcher and this could be a topic for future investigations.

It has also been found that the frequency of the stimulus, at least in single molecule experiments, is determinant to produce unfolding processes [50]. This effect could be related to a stochastic resonance process in which a stimulus provided at the right frequency, even if its amplitude is very small, in the presence of a random noise, is able to induce effects that could be produced by a perturbation stimulus only at high amplitude. Moreover, the unfolding and refolding process is insensitive to random mechanical noise whereas it is sensitive to a specific range of frequency used to cyclically stimulate the molecule. Typically, for a system whose physics can be described using a potential energy curve with two minima, there can be a synchronization for the jumping between the two states with the cyclic stimulus at low amplitude. For a specific frequency of the stimulus, related to the Kramers theory and to the activation energy, a time-scale matching condition can be obtained. Accordingly, the process of folding/unfolding, which in the specific case has been tested for talin and it is thus strongly related to mechanosensitivity phenomena, occurs under the control of a sort of band-pass filter. It is interesting to speculate that small amplitude signals provided at a

specific frequency, even high frequencies that are generally considered not biologically relevant, could affect the function of biological systems [51]. These analyses raise the question of the significance of single-molecule experiments in representing much more complicated physiological aspects. Single molecule experiments are at the basis of the initial comprehension of the mechanosensing and mechanotransduction of cells, as in the case of the binding of vinculin to partially unfolded portion of talin molecules [45]. For example, single molecule experiments have clarified that the lifetime of a bond depends on the applied force and the rupture force strongly depends on the rate of force increase. Moreover, analytical models of the molecular clutch model, which has been validated by many experiments, are based on parameters obtained from single molecule experiments. At the same time, in recent years in the literature, works showing that, inside single cells, conformational changes of proteins subjected to mechanical forces occur and that these processes can be relevant for cell behavior, have appeared. For example, in a work by Johnson *et al* [52], the authors found that mechanically relevant proteins exposed otherwise buried cysteines when cells were subjected to shear stress. These processes could be the starting signal for biochemical cascades inside cells [53]. The role of talin unfolding for a regular mechanotransduction process, such as focal adhesion growth and YAP translocation to the nucleus, in living cells has been also verified by altering its conformation flexibility [35]. In this context it is important to stress that the altered protein flexibility was confirmed by single molecule experiments. Other experiments showed the possibility of determining the loading rate of mechanically relevant proteins in living cells [54] and these techniques pave the way for an important comparison between experiments performed with single molecules and experiments on living cells. The use of molecular probes for internal forces experienced by molecules in living cells, typically based on the FRET technique [55], and their development will certainly allow to better close the gap between single molecule experiments and experiments on living cells.

To summarize, we can establish an analogy between cells growing on substrates of different rigidity and the rate of force increase that we can control in a cell stretching experiment. If cells are growing on a soft substrate (in the order of 1 kPa), applying low rates of force increase does not affect how they interact with the substrate. This means that cells do not develop large focal adhesion complexes and tend not to spread too much over the surface. If high rates of force increase are applied, there are two possibilities: or the mechanosensing apparatus is not activated before the integrin molecules disengage from the substrate, or the apparatus, via the partial unfolding of the talin molecule is activated and the adhesion areas are reinforced [35, 56]. In the latter case, the behavior of cells is similar to what happens when they adhere to a stiffer support with the activation, after some minutes from the beginning of the stimulus, of the GTPase RhoA and the formation of actin stress fibers.

In living systems, cells are exposed to many different stimuli at the same time, such as chemical and mechanical ones, and it is impossible to disentangle the role of each specific signal by experiments performed *in-vivo*. The use of cell stretching devices may help to decouple the different cues and identify the effect of individual stimuli [57, 58]. As an example, in the heart, cardiac fibroblasts are typically quiescent cells, but they can be activated to myofibroblasts by an abnormal stretching stimulus [59]. However, it is still a matter of debate if the activation is a consequence of the mechanical stimulus, the release of chemical messengers from the ECM, for example TGF-$\beta$, or a combination of both effects. The use of stretching devices could help understand the role of pure mechanical stimuli.

Mechanotransduction also depends on the orientation of the applied forcing stimulus. For example, it has been shown that a mechanotransduction process could be particularly efficient when the force is applied parallel to the binding interface in the case of T-cells [60]. The exploitation of a biaxial stretch with independent control of the two stretching directions could elicit the application of forces with any specific direction in a 2D system such as a cell monolayer. The different effects obtained by a different directionality of the applied force could explain the reorientation of the cells along specific directions when anisotropic mechanical stimuli are provided (see section 5.1). Single-molecule experiments revealed that the typical reinforcement effect due to vinculin in adhesion complexes is present only when forces are applied along specific directions with respect to the geometry of the complexes. This mechanism could reinforce adhesion complexes only along specific directions while destabilizing other complexes, producing in this way a reorientation of cells, as typically occurs in tissues exposed to cyclic deformations.

Mechanobiology relies also on the effect of forces applied in the plane of the plasma membrane. In this case the 'force-from-lipids' effect could be related to the stretching of cells. Once it has been demonstrated that the stretching of the support on which the cells adhere is transmitted to the plasma membrane, the relevance for mechanobiology is linked to the fact that the stretching of the lipid bilayers has been shown to affect the behavior of mechanosensitive channels [61–63]. By coupling cell stretching with $Ca^{2+}$ imaging, the opening of mechanosensitive channels as a consequence of cell and plasma membrane stretching has been demonstrated [63–65]. These kinds of experiments are particularly relevant in the case of the cardiac tissue, where the electromechanical coupling involves the continuous cyclic stretching of cells [66]. Ultimately, the

mechanism is the same one that has been applied for a long time with patch-clamp measurements, where a pressure step is applied to a membrane patch containing ion channels causing them to open. The use of channel blockers, such as the chemical inhibitor GsMTx4 or $Gd^{3+}$ ions [67], can be exploited to further demonstrate that the variation of $Ca^{2+}$ influx upon cell stretching is directly related to the opening of ion channels. At the same time, the frequently observed increase in proliferation of cells upon their exposure to mechanical cyclic stretching, has been related to the increased activity of mechanosensitive channels, in particular the Piezo1 channel [68, 69].

Most of mechanotransduction effects are related to signals directly transmitted to the cell nucleus from the extracellular membrane exploiting adhesion complexes, the cytoskeleton and all the complexes linking the cytoskeleton to the nucleus, such as the linker of nucleoskeleton and cytoskeleton (LINC) complexes [70–79]. At the same time, these complexes allow the nucleus to detach from the cytoskeleton in case of a rapid and intense stretch [56, 80]. By exploiting systems based on the attachment of magnetic beads to integrins on the membrane of living cells, it has been demonstrated that a periodic pulling of these beads affects the transcription processes inside the nucleus [75]. Even if the transmission of the mechanical signals from the cell membrane to the nucleus appears a clear mechanistic phenomenon, how the mechanical signals reaching the periphery of the nucleus are translated into biochemical pathways is not clear, especially when the mechanical cues confer a sort of memory to the cell. The underlying mechanisms could be related to a partial decondensation of the chromatin inside the nucleus (changing the ratio between the amount of heterochromatin and euchromatin) [72, 79, 81, 82] or to the stretching of the nuclear membrane leading to pore opening, thus altering the distribution of epigenetic/transcription factors between the cytoplasmic and nuclear regions. Noteworthy, both mechanisms result in a change of gene expression inside the nucleus. Another possibility is that of a decrease in the viscosity of the region inside the nucleus upon cell stretching, leading to increased protein diffusivity [83]. Considering all these aspects, the development of cell stretcher devices coupled with high resolution optical live cell imaging could greatly contribute to reveal the mechanobiology of the force transmission inside the nucleus [84]. It has been demonstrated that effects on the nucleus due to cell stretching are very fast, in the order of seconds to a few minutes and, accordingly, the time to refocus the sample after a stretching step, if needed, should be very rapid [71, 85]. In this way it could be possible to observe the changes induced inside the nucleus, for example in terms of chromatin condensation or decondensation, and the reorganization of the actin cytoskeleton to protect the nucleus from excessive DNA damage. Indeed, it has been suggested that the cytoskeleton can operate as a safeguard against excessive mechanical stresses [80]. Apart from the fluidization effect already discussed and occurring for high strain rates and the detachment of the connections between the nucleus and the cytoskeleton, the cytoskeleton can reorganize around the nucleus, forming a sort of protective cap. If we consider the effect of stretching on mechanosensitive channels in the membrane, changes are expected in the time interval of ms after the application of the deformation. In this case, an automatic focusing and tracking system would be highly desirable. To follow changes to the nucleus immediately after the application of a mechanical stimulus, Horvarth *et al* implemented an isotropic stretcher device exploiting vacuum and coupled with a focusing follower system assuring also an in-plane tracking [86]. In their work, they were able to obtain information on the nuclear decoupling from the cytoskeleton as a function of the applied strain rate.

## 3. Cell stretching devices

Here we will initially provide an overview of the different cell stretching systems that have been developed to date. Then, we will focus on technical aspects of the specific devices that we developed in-house.

Different cell stretching devices have been exploited for mechanobiology investigations. Most of them are custom-made devices, although some systems are also commercially available [87, 88] (table 1).

Regardless of the purchase cost, the major advantage of home-developed devices is the high versatility in terms of the periodic signals that can be applied, the possibility of coupling the stretching signal with other stimuli such as a fluid flow or electrical stimulation, or of adapting the device for time-lapse optical microscopy.

The general scheme of a mechanical stretching device is composed by a deformable chamber for culturing cells and by clamping parts to transmit mechanical signals in a controlled way. We can classify the different types of stretching devices based on the directionality of the stretching signals that they can provide and on the actuation method. The main typologies of stretching geometries are uniaxial [18, 89–91], biaxial [92–95] and radial (isotropic) [96, 97] (figure 2). It has been demonstrated that different straining geometries produce different effects on the same type of cells [98]. In the case of uniaxial stretchers, in addition to the elongation of the substrate in the direction of the actively induced stretching, there will also be a contraction in the perpendicular directions due to the Poisson effect, i.e. the volume conservation of the stretched material (we can typically assume a bulk Poisson ratio of 0.5 for elastomers such as PDMS). The

**Table 1.** List of some companies producing commercial cell stretching devices. In each case the actuation method and the webpage of the company are reported.

| Commercial devices | Actuation method | Web page |
|---|---|---|
| Flexcell (from Flexcell International) | Pneumatic | www.flexcellint.com/ |
| StrexCell (from STREX Inc.) | Motor actuation | https://strexcell.com/ |
| Curibio Cytostretcher | Motor actuation | www.curibio.com/cytostretcher |

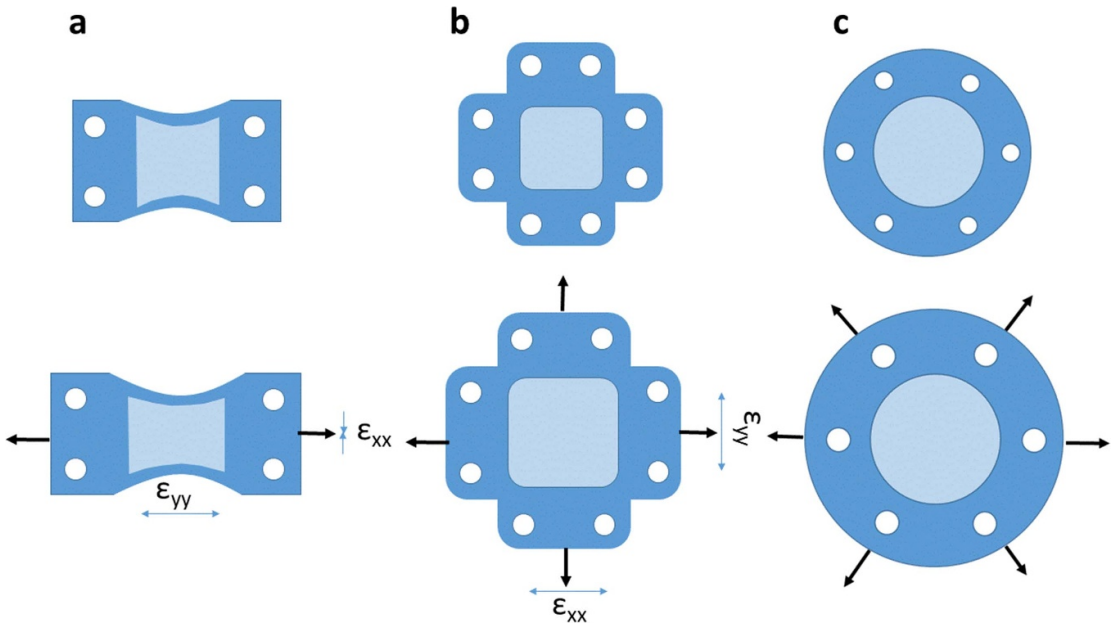


**Figure 2.** Scheme of the different stretching configurations and elastomer supports that can be exploited: (a) uniaxial stretching where, together with a positive strain in the stretching direction, a negative strain in the perpendicular direction is obtained (the specific geometry has been optimized to produce an almost pure uniaxial deformation); (b) biaxial stretching with the independent control of the two perpendicular stretching directions; (c) isotropic stretching obtained by stretching along six symmetric directions. Completely isotropic stretching conditions could be obtained by pneumatic actuation with a circular region in which the vacuum is controlled.

in-plane deformation (it is to be stressed that also a deformation of the thickness is present and it typically produces a vertical shift of the focus position complicating the conditions of continuous image acquisition) is accordingly always biaxial with strains of opposite sign along the two principal directions when only one active stretching direction is present. The ratio between the strains in the two in-plane perpendicular directions is, in ideal conditions, provided by the Poisson ratio. However, in real experiments, it is partly controlled by the geometry of the polydimethylsiloxane (PDMS) chamber and its clamping to the stretcher device [99]. This is due to the presence of structures like the borders of the culture chamber which confer directional asymmetries to the system. In many cases it can be useful to perform a FEM simulation of the strain map to know the distribution of the strain values in the support (see section 4).

Biaxial stretchers are able to apply deformations to the PDMS substrate in two perpendicular directions. In this case, it is possible to have at the same time positive elongations in both directions. In the literature, there are many examples of biaxial cell stretchers exploited to study different cell types [65, 96, 100, 101]. However, the number of studies in which non equibiaxial stretching protocols or out-of-phase stretching in the two directions are applied are scarce. In the case of isotropic stretchers, the elongation is applied at the same time in all directions (or in six equidistributed radial directions) and they are typically exploited to reproduce deformation stimuli experienced by cells residing in hollow organs like the heart or lungs. The deformations can be obtained by exploiting different actuation systems (see table 2 for a list of the main actuation systems with their advantages and disadvantages). One possibility is represented by pneumatic stretching devices [95, 102–104] (figure 3). In this case, both negative and positive pressures could be exploited to decrease or increase the volume of a deformable flexible chamber. The volume change of the chamber produces a measurable deformation of the substrate to which cells are adhering. The pneumatic actuation allows to obtain uniaxial, biaxial and radial strains of the substrate.

Other actuation systems are based on the use of stepper motors, electromagnets and voice coils actuators, where a mechanical movement of an arm is transmitted to the elastomeric substrate by a simple linear

**Table 2.** Main actuation methods exploited in cell stretcher devices.

| Actuation method | Advantages | Disadvantages |
|---|---|---|
| Motor actuation (elongation by stepper motors) | Easy to implement<br>Very good control over strain amplitude and rate<br>Easy to implement, in the uniaxial stretching configuration, with more than one support | Size of devices is quite large<br>Difficult miniaturization<br>Possible contamination from the nearby motors<br>Not always possible to insert into a cell incubator |
| Motor actuation (by indentation) | Very good control over strain amplitude and rate<br>Easy to implement with more than one support | In some cases, needs lubricants<br>Not easy to implement at high strain rate<br>Not easy to couple with optical microscopy for real time imaging |
| Pneumatic actuation | Easy miniaturization and implementation with microfluidics<br>Can work with the stretching device far from the pneumatic control system | Not easy to control the exact strain map<br>Not easy to implement at high strain rate<br>In some cases, needs lubricants |
| Dielectric elastomer actuator (DEA) | Can apply high strain rates<br>Can be integrated within the culture substrate<br>It can be easily miniaturized. | Possible effects of strong electric fields on cells<br>Deformation limited to the region with the non-transparent electrodes |
| Electromagnetic and voice Coils actuators | Can apply high strain rates<br>Easy to couple with real time optical imaging | Possible effects of electric and magnetic fields on cells<br>Possible problems with heat coming from the excited coils<br>Not easy to have linearity |

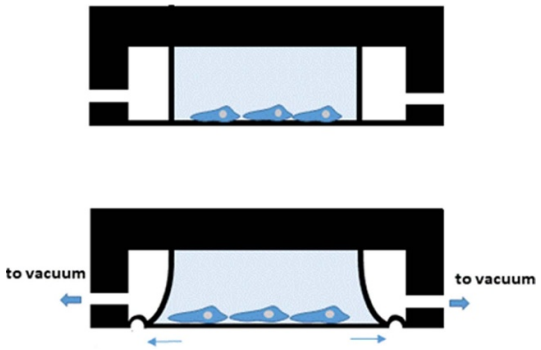


**Figure 3.** Typical scheme for pneumatic stretching devices. A vacuum performed in the lateral chamber of the system allows the extension of the membrane on which cells are adhering.

translator arm or by the presence of a magnetic material embedded in the substrate [105–108]. This method is very simple to implement, especially if stepper motors are used, and offers the possibility of driving the devices by easily programmable low-cost microprocessors such as Arduino [30]. Another possible actuation system is based on the use of dielectric elastomer actuators (DEAs) which allow the application of very fast strain deformations (sub-ms resolution) and at the same time can be compatible with optical inspection [109, 110]. In this case, an elastomer such as PDMS is sandwiched between two stretchable electrodes and the application of a high voltage potential difference induces the approaching of the two electrodes and the stretching of the elastomer. Another advantage of DEA-based stretchers is that they can be easily miniaturized to make them compatible with other instruments such as incubators and optical microscopes. A problem linked to this actuation system is the exposure of cells to strong electric fields which can affect, at least for some cell types, their behavior.

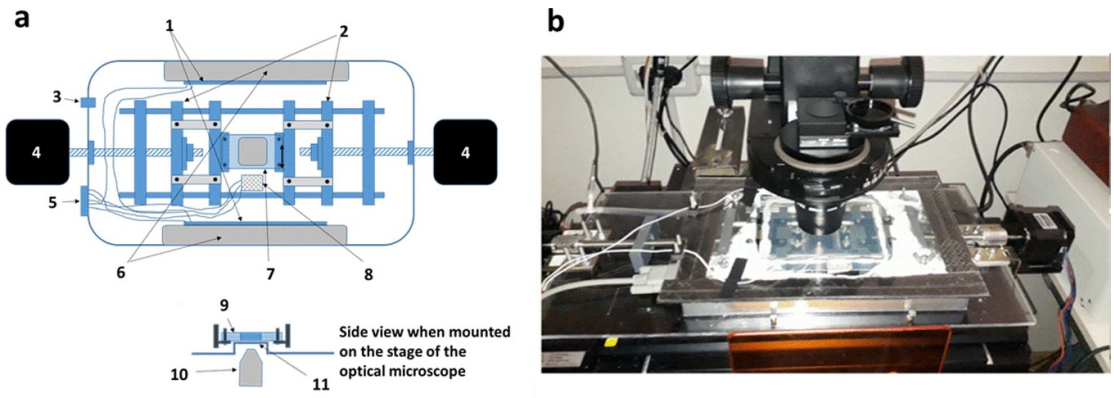


**Figure 4.** (a) Scheme of a uniaxial cell stretcher positioned inside an on-stage cell incubator. A side view is also reported to show the position of the microscope objective with respect to the PDMS chamber used to stretch the cells. 1- Thick film resistors; 2- movable barriers; 3- $CO_2$ inlet; 4- stepper motor; 5- electrical connections; 6- water reservoirs; 7- stretchable PDMS support; 8- temperature and relative humidity sensor; 9- PDMS chamber; 10- objective; 11- glass window; (b) image of the stretcher mounted on the stage of an inverted optical microscope.

In the following sections, we will provide some construction details of the different cell stretching devices that we assembled in our lab. Other laboratories interested in adopting these techniques, should possess or develop abilities related to a mechanical workshop to construct the device and to programming of microprocessors which are able to drive for example stepper motors to design different stretching protocols with different and possibly arbitrary signals. The use of a mechanical workshop is mandatory also for the production of molds of elastomeric supports. If the labs are willing to couple imaging with optical microscopes to the devices, the system should be positioned inside an on-stage cell incubator, or incubators surrounding the microscope should be adopted, to assure stable conditions for cell populations. To compare the results of specific experiments with those obtained from other labs, it is important to know exactly the strain map of the elastomeric support and, accordingly, abilities in calibration procedures to measure the strain map should be developed.

### 3.1. Uniaxial cell stretching device

In this section we will describe the construction of a fully-automated uniaxial cell stretching device integrated with time-lapse imaging by optical microscopy. Whereas the mechanical aspects of the specific cell stretching devices are different in the cases of uniaxial, biaxial and isotropic, the driving interface can be similar and the integration with the time-lapse functionality is obtained exploiting the same type of on-stage cell incubator that we previously developed [30]. Therefore, the on-stage cell incubator setup will be briefly described only for the uniaxial stretcher.

Figure 4(a) shows a schematic of the uniaxial stretching device which relies on the symmetric actuation by means of two stepper motors. The stretcher can provide up to 20% strain to a PDMS substrate by using two screws connected to movable bars. All the system is positioned inside an on-stage cell incubator to allow time-lapse imaging of the cells by an inverted optical microscope during the stretching experiment (figure 4(b)). The on-stage incubator is made of a polymethylmethacrylate (PMMA) base and an aluminum side wall. The PMMA base is provided with a glass window just below the PDMS chamber to improve the optical transparency. The top cover of the incubator exploits a transparent indium-tin-oxide (ITO) window and an electrical current, controlled by a PID feedback of an Arduino board, avoids water vapor condensation. The temperature inside the chamber is controlled by two thick film resistors (TFRs) maintaining the temperature at a value slightly lower than 37 °C; the final temperature of 37 °C is achieved by means of the small amount of heat generated by Joule effect produced by the flowing of the electric current through the ITO window. The temperature inside the incubator is measured by a DHT22 sensor and constantly compared to the reference setpoint of 37 °C. The same sensor also measures the relative humidity in the chamber which is mainly ascribable to the evaporation of two water reservoirs in direct contact with the TFR elements. More details of this system can be found in [111]. The stepper motors are controlled by an Arduino microprocessor unit interfaced with LabVIEW software. Different stretching protocols can be applied and specific waveforms can be obtained by implementation of a home-developed Python software (see SI). The general protocol is based on the assignment of a variable time interval between successive steps of the stepper motors. The intervals are determined by the analysis of the periodic function and a function inversion process that assigns different time intervals to equal spatial deformations, the latter corresponding

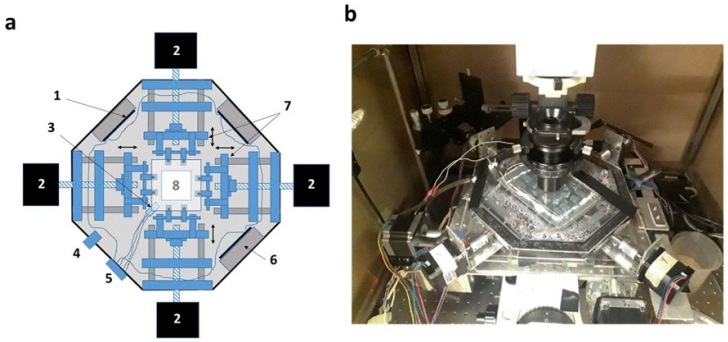


**Figure 5.** (a) Scheme of a biaxial cell stretcher positioned inside an on-stage cell incubator. 1- Thick film resistor; 2- stepper motor; 3- temperature and relative humidity sensor; 4- $CO_2$ inlet; 5- electrical connections; 6- water reservoirs; 7- movable barriers; 8- PDMS support; (b) image of the stretcher mounted on top of a motorized stage of an inverted optical microscope.

to the deformation produced by a single step of the motor. In this case, the maximum rate of deformation is given by strain due to a single step divided by the minimum time interval between two successive steps for these motors (about 4 $\mu$s). The detailed protocol for the reproduction of every waveform has been reported elsewhere [30]. All the system is coupled to a home-developed automated microscope stage which can move in the two lateral directions by means of two stepper motors. To obtain time-lapse imaging, the stretching system is coupled to the motorized microscope stage and both are controlled by the same Arduino Mega. A certain amount of time for the cyclic stretching part is established and, at the end of each time interval, a series of images, with the stretching part turned off (at the minimal displacement position), is acquired in different positions of the sample. Accordingly, the system cannot be defined properly as a 'live-imaging' system because cells are imaged when the stretching is temporarily stopped. Below, we will introduce some attempts that have been recently done in the literature to limit this issue and obtain continuous imaging of the stretched support even in the case of high-frequency stretching.

### 3.2. Biaxial cell stretching device

Figure 5 shows the scheme and an image of a biaxial stretcher we recently developed. Interestingly, by means of biaxial stretching mode it is possible to simulate the sudden occurrence of a damage in a vessel or other parts of the vascular system. In this context, typical studies in the literature focus on the kinetics of cell reorientation starting from a resting situation where cells are not exposed to any cyclic stretching (see section 5.1). However, it should be underlined that in *in-vivo* scenarios, the reorientation process upon damage occurs when cells have reached their homeostatic condition subjected to cyclic stretch. This condition typically implies a decrease of the morphological parameter of circularity and a concurrent increase of cell elongation in a specific direction compared to an unstimulated cell. In our home-built setup, we are able to set an initial period for cell equilibration in the presence of a cyclic stretching protocol along one direction, and then, to immediately apply a stretching protocol along a different direction. We performed several experiments in which the stretching direction was rotated by 90° and we followed the kinetics of cells reaching a new homeostatic equilibrium (see section 5.1 for some recent results from our group). Similar experiments have been performed on endothelial cells that line the internal surface of blood vessels [112]. These cells are continuously exposed not only to a circumferential stretching but also to a pulsating shear strain due to the rhythmic pumping of blood flow. Both stimuli contribute to the orientation of endothelial cells along the main axis of vessels. Concerning the shear stress, the activation of inflammatory signals when the flow direction is rapidly changed after a long period of time in which cells have oriented their stress fibers and reached a homeostatic condition, has been demonstrated [113]. Accordingly, it can be expected that a change of the stretching direction after stress fibers aligned perpendicularly to a prolonged oriented stretching stimulus, can activate inflammatory and atherogenic signals as well. Indeed, Kaunas *et al* [112] demonstrated that a change of the stretching direction by 90° after a prolonged stretching stimulus had aligned endothelial cells' stress fibers, produced an activation of c-Jun N-terminal kinase (JNK), which is implicated in the early stages of atherosclerosis development. After about 6 h of the new cyclic stretching protocol, the activation of JNK went back to the previous level. In the case of equibiaxial stretching, where the cells are not able to reorient, the level of JNK does not change upon a change of the stretching protocol. This elegant study clearly highlights that the reorientation process is a particularly important physiological event when cells reached a homeostatic state concerning cyclic stretching and a change in the stretching direction occurs, for example as a consequence of a tissue damage. Therefore, it is of paramount importance

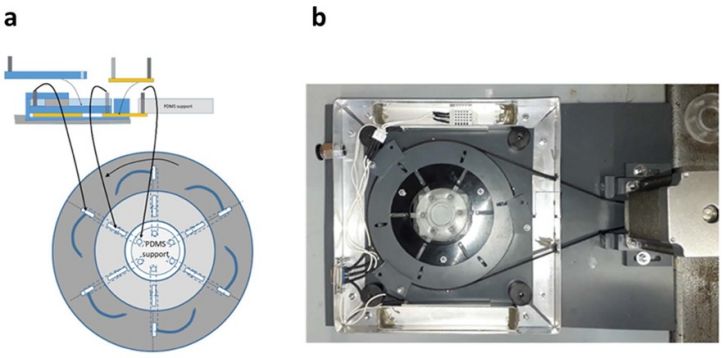


**Figure 6.** (a) Scheme of our isotropic stretcher showing a cross section of the transmission system (top) and of the top view including the PDMS support (bottom); (b) picture of the isotropic stretcher included inside the on-stage cell incubator.

for these kinds of mechanobiology studies to rely on stretcher devices that allow to generate highly specific stretching protocols along different directions.

As can be observed in figure 5(b), the stretcher has been positioned inside an on-stage cell incubator, whose working principle is similar to the one previously described for the uniaxial stretcher. In this case, however, the temperature/humidity sensor is not positioned close to the cell chamber and, due to temperature inhomogeneity inside the incubator (related to the presence of the glass covered with ITO) a calibration has to be performed using a thermocouple placed directly inside the chamber. The device is controlled by a LabView interface allowing independent stimulation of the two axes. To obtain this, an Arduino microprocessor controls two motor drivers (DM542) each one driving two stepper motors (NEMA 17). While in the uniaxial device a single mechanical stimulus is provided by two stepper motors working with the driving current in series, here two couples of stepper motors are involved to independently apply two mechanical stimuli. Thus, the original Arduino firmware used for the uniaxial device has been deeply modified. The uniaxial stretcher software architecture was based on simple delay times between consecutive steps of the motors. This approach is no more suitable if additional mechanical stimuli must be imposed to additional stepper motors. Thus, the Arduino routines dedicated to the mechanical stimuli have been completely rewritten. The delay function has been substituted with priority interrupting functions which assure the simultaneous application of the mechanical stimuli in the $x$ and $y$ axis. Each couple of stepper motors controls the stretching in one direction and the two couples can be operated with protocols having different maximum amplitudes and different phases (see movie S1 and S2).

### 3.3. Isotropic cell stretching device

Deformations of hollow organs undergoing cyclic stretching, such as heart, vessels and lungs, can be better described and reproduced by considering isotropic strains, even if the geometry of physiological stretching is commonly more complex and anisotropic. The quasi-isotropic deformation is mainly due to the action of a hydrostatic pressure acting quasi-radially on the internal surface of the organ. Moreover, in the case of the cardiac tissue, when considering cell stretching of the plasma membrane, it should be noted that there are different sections of the membrane having mutually perpendicular orientation. Here, a simple uniaxial stretching geometry is clearly not able to recapitulate the behavior of all the different sections of the membrane at the same time [114]. In the literature, several strategies have been pursued to get an isotropic stretching, including an annular pneumatic system or the deformation of a planar membrane by an indenting ring [106]. Our home-built system takes inspiration from the IsoStretcher device [96, 114]. The stretching of a circular symmetric PDMS substrate occurs by moving six rods with directions separated from each other by 60° (figure 6). A rotational movement of a disc with specifically designed guide grooves produces the radial movement of the rods. By changing the geometry of the grooves, different external translations (i.e. strain) from the same rotational movement can be obtained. This is important when the stretching rate is to be increased, considering the limiting value of the time resolution for consecutive steps of the stepper motor. As shown in figure 6(a), the stretcher device is integrated with an on-stage cell incubator with the stepper motor left outside. The construction strategy of the cell incubator is similar to the one used in the case of the uniaxial stretcher [30, 111]. In the SI (figure S2) other details of the different parts of the device are shown.

### 3.4. Supports for cell stretching experiments

PDMS is the most widely used elastomer in cell stretching devices due to the fact that it is cheap, optically transparent, permeable to gasses and non-toxic when fully cured. Three different types of PDMS substrates

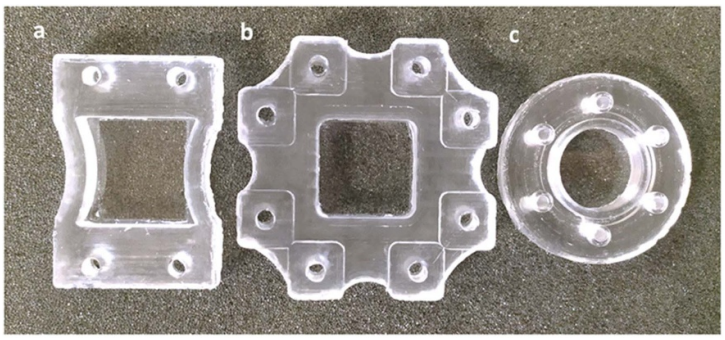


**Figure 7.** PDMS supports used to perform cyclic stretching experiments: (a) support for the uniaxial stretching device; (b) support for the biaxial stretching device; (c) support for the isotropic stretching device. The supports are prepared by exploiting negative molds obtained by CNC milling of a PVC base. To improve the optical microscopy imaging, the milled region where cells are seeded is covered with a thin cover glass in order to have a thin and smooth surface in the corresponding PDMS region.

we use for the different stretchers are shown in figure 7 (see figures S3–S5 for further details of the supports). Just prepared PDMS substrates are highly hydrophobic (contact angle of about 105°–110°) and, as such, they do not promote cell adhesion without further functionalization. PDMS surface can be treated with an air or oxygen plasma discharge to greatly increase its hydrophilicity and to make it more prone to coating with proteins of the ECM such as collagen, fibronectin or laminin, thus promoting cell attachment [115, 116]. PDMS substrates are easily obtained by mixing a pre-polymer and a curing agent, their ratio determining the stiffness of the cured support. In order to obtain mechanically stable supports for cell stretching experiments, a 10:1–20:1 ratio is typically used, assuring a stiffness in the order of a few hundreds of kPa. However, this type of support is still too rigid compared to human soft tissues; if the specific experiment to be performed requires cells to adhere to softer supports, a layer of a softer elastomer or hydrogel (Young Modulus from a few kPa to 30 kPa) can be added on the more rigid PDMS. This modification paves the way for instance to the coupling of cell stretching experiments with TFM, which allows the investigation of the effect of mechanical stimuli provided by the stretcher on the force applied by cells on the support (see section 6). Different types of PDMS polymers can be exploited in this case (for example PDMS Sylgard 527, assuring the lowest rigidity of about 5 kPa) [117]. Alternatively, a layer of soft polyacrylamide (PAA), whose rigidity can be finely tuned, can be exploited. In each case, the rigid attachment of the softer layer to the underlying support should be verified [29]. However, when using PAA it should be reminded that the obtained hydrogel, once put in a liquid environment and undergoing a stretching deformation, according to a poroelastic model, will produce a partial outflow of the water entrapped in the hydrogel which can eventually impact on cell behavior and fate. Indeed, it has been demonstrated that the fracture in epithelial cells sheets could be due to an increase of the hydraulic pressure during stretch [97].

Apart from PDMS based supports, other types of substrates have been developed. In particular fibrous scaffolds allow the reproduction of surfaces more similar to the native ones for cells of the tendon or muscle tissues [118–120]. In fact, they introduce an anisotropic environment for cells, both topographically and mechanically, and they are relevant to understand separately the role of topographic anisotropy and mechanical stress on the alignment mechanism of cells. In many cases, these supports can be produced by exploiting the electrospinning technology producing also 3D structures. Another interesting substrate that has been considered for stretching experiments is based on decellularized plant biomaterials [121]. In fact, their elasticity allows the possibility of a strain up to 10%–20% and their 3D structure resembles that of human vasculature with interconnected porosity for nutrient diffusion. In the case of stretching devices for 3D cell studies, hydrogels structures, optimized to allow cell cultivation (for example adding alginate to reduce the problem due to hydrogel shrinking), are exploited to deliver mechanical signals to cells [21].

## 4. Calibration of the strain and validation by FEM simulations

When cells are exposed to cyclic stretching, the reorientation process they undergo in response to the perceived mechanical stimulus is one of the most investigated behaviors. The process, which involves a reorganization of the cytoskeleton and its focal adhesion complexes, is usually studied in terms of the final equilibrium position reached by the cells but also in terms of the dynamics underlying the process itself. The mechanical properties of the deformable substrate and the characteristics of the applied stretching signal are usually key factors to be considered when studying reorientation. The main observables of the reorientation process are the direction of equilibrium orientation, an angle that is extracted by analyzing the orientation of

a significant number of cells after a period of stretching perturbation, and the spreading around this direction.

Several theories have been developed to explain how the polarization direction can be related to the physical properties of the substrate, finding in the strain map imposed onto the surface of the substrate the most important macroscopic property to be related to cellular behavior [122, 123]. Therefore, an accurate knowledge of the strain distribution over the substrate surface, in order to compare theories with experiments and experiments performed with different devices in different labs, is crucial. Accordingly, the first step in the exploitation of cell stretchers is the calibration of the strain, ideally in every position of the substrate. Experimental calibration involves the observation by an optical/fluorescence microscope of fiducial spatial markers, such as micron-sized beads or small defects on the surface of elastomeric substrates, and exploitation of image post-processing analysis, such as, for example, the use of particle image velocimetry (PIV) software. While observing the entire surface of the substrate might be desirable, doing so is also a cumbersome and time-consuming task. For this reason, the area of interest for calibration is typically just the central region of the substrates, at least when large substrates are used. FEM simulations are a powerful tool to virtually extend the analyzed area to the whole substrate surface if the precise boundary and clamping conditions for the substrate are known. FEM could also be exploited to optimize the substrate design. In literature, cells are proven to respond to strains in the range 4%–20%, whereas strains far beyond this value usually end up in cell detachment. Below this threshold they are ineffective, probably because the main physical parameters cells respond to, i.e. the loading rate, cannot reach a threshold value [124].

In our devices, experimental calibration was carried out by gradually incrementing the deformation of a 20:1 PDMS substrate at chosen step values while observing either small imperfections in the PDMS surface or micron-sized beads seeded on the surface by the optical microscope. 10x or 20x objectives were used to acquire images of approximately 1 $mm^2$ area, located at the center of the PDMS substrate, either in Phase Contrast or fluorescence acquisition mode. After the acquisition, images were processed with ImageJ into stacks and aligned with the Template Matching plugin with respect to an arbitrarily chosen non-moving point. The $x$ and $y$ coordinates of the points observed during calibration were exported from ImageJ analysis and then analyzed with a custom-developed Python script.

The theoretical basis for the calibration process is derived from continuum mechanics theory [30]. Briefly, a $2 \times 2$ matrix representing the substrate deformation and defined as the sum of an identity matrix and the matrix of the deformation is calculated exploiting the position of triads of small features in the image. The four unknowns of the deformation matrix are evaluated considering the deformation of two sides of two triangles according to the expression:

$$\begin{cases} \mathrm{d}\boldsymbol{x}_1 = \begin{pmatrix} F_{11} & F_{12} \\ F_{21} & F_{22} \end{pmatrix} \mathrm{d}\boldsymbol{X}_1 \\ \mathrm{d}\boldsymbol{x}_2 = \begin{pmatrix} F_{11} & F_{12} \\ F_{21} & F_{22} \end{pmatrix} \mathrm{d}\boldsymbol{X}_2 \end{cases}$$

where $x$ and $X$ are defined as in figure S6 and we are considering small deformations. The diagonalization of the matrix $\boldsymbol{F}$ allows to determine the stretching along the two principal directions according to:

$$\boldsymbol{F} = \begin{pmatrix} \lambda_1 & 0 \\ 0 & \lambda_2 \end{pmatrix}$$

in which $\lambda$ is defined as $\lambda = \left|\frac{\mathrm{d}\boldsymbol{x}}{\mathrm{d}\boldsymbol{X}}\right| = \frac{L+\Delta L}{L}$, whereas the strain $\varepsilon$ is defined as $\varepsilon = \frac{\Delta L}{L} = \lambda - 1$. The matrix $\boldsymbol{F}$ is in principle the result of a rotation and a deformation process (the two operations are not commutative and would require different deformation matrices if the order is changed). By separating the two contributions using polar decomposition, it is possible to evaluate the presence of a rotational transformation that would imply the presence of shear deformation. We can anticipate that in all the calibration steps we performed for the different stretching devices (uniaxial and biaxial) we found a rotation angle almost equal to zero and we can accordingly neglect the presence of a shear deformation.

To perform the calibration procedure, we developed a script that evaluates the evolution of the features on the PDMS surface—which are usually taken in triads of points for an easier-to-visualize approach—while increasing the applied deformation. The associated deformation matrix is then calculated. Alongside the experimental calibration, FreeCad designs of the PDMS substrates were virtually stretched in FEM simulations. In the following Section the results of both experimental and simulated calibrations for the uniaxial stretcher are reported. The procedures for the calibration of the biaxial and the isotropic stretchers are reported in the Supporting Information section.

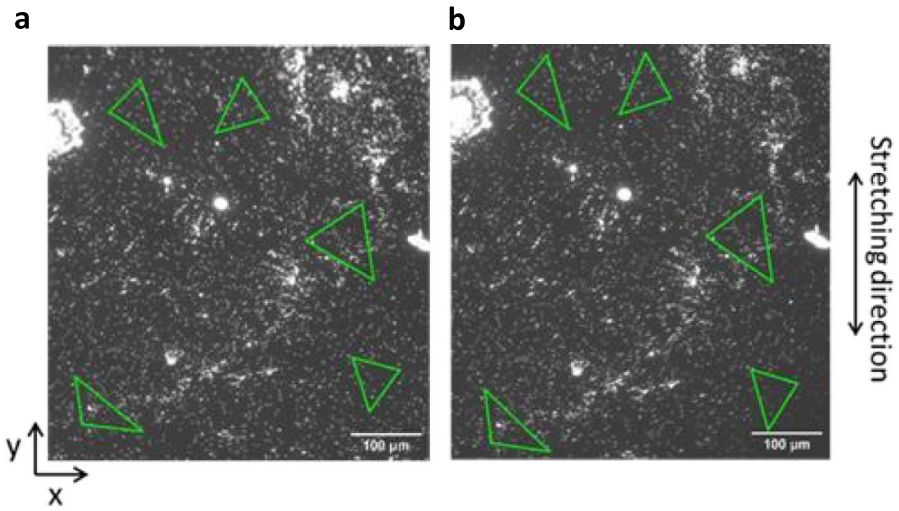


**Figure 8.** Images of fluorescent nanobeads deposited on the PDMS substrate for the calibration of the uniaxial stretcher strain: (a) substrate not deformed; (b) substrate after deformation. The green triangles highlight the triads of beads for the stretch calibration procedure.

### 4.1. Calibration of the uniaxial stretcher device

The surface of a PDMS substrate of the uniaxial stretcher was treated with 5% v/v APTES in ethanol and a suspension of fluorescent red carboxylate-modified polystyrene nanobeads in Tris buffer 100 mM at pH = 8.5 (Latex beads, carboxylate-modified polystyrene, fluorescent red—aqueous suspension, 0.5 $\mu$m mean particle size). The substrate was observed with a 20x objective (NA = 0.4) while being deformed along the $y$-axis by the stepper motors (see figure 8). The values of $\varepsilon_{xx}$ and $\varepsilon_{yy}$ at different steps, as obtained from the Python script, are plotted against the number of applied steps of the motor (200 steps/rev) (figure 9(a)). Whereas $\varepsilon_{yy}$ has a positive sign, and increases with the steps, $\varepsilon_{xx}$ describes a small compression, hence the negative sign, occurring along the perpendicular direction $x$. This compressive effect is in agreement with the Poisson effect and a conservation of the PDMS volume. The value of the overall strain achieved, $\varepsilon$, is plotted in figure 9(b). It is important to stress that once the principal strain values are known, it is possible to find the directions of zero strain, as shown in figure 9(c). This is an important parameter because, according to some theories, cells align along these directions when subjected to cyclic stretching (see section 5.1 and SI, equation (S14)).

Experimentally relevant conditions were met for an overall strain value close to 6%–7% ($\varepsilon = (6.6 \pm 0.5)\%$). In figure S7, by exploiting the PIV software, images showing the substrate displacements in false colors are reported.

Alongside the experimental calibration, FEM simulations were performed recreating the deformation of a 20:1 PDMS substrate (Young Modulus ($E$) = 0.0007 GPa, Poisson ratio ($\nu$) = 0.499, density ($\rho$) = 1090 kg m$^{-3}$) along the $y$ axis by ∼2.04 mm per side, as depicted in figure S8. From the results of the FEM simulation, the average values of the strain components $\varepsilon_{xx}$ and $\varepsilon_{yy}$ over a central square of semi-side $L$ are extracted and plotted as a function of $L$ (figure 10). The behavior of $\varepsilon_{xx}$ and $\varepsilon_{yy}$ follows the same trend of the experimental data, namely a compression and an elongation contribution, respectively. The corresponding overall strain, $\varepsilon$, is plotted in figure 10(b). The situation closer to the experimental one corresponds to $L = 0.1$ cm, where the value of strain is $\varepsilon = (7.84 \pm 0.01)\%$.

To visualize the data from the FEM simulation, in figure 11 the overall strain $\varepsilon$ is presented as a contour map with, superimposed, one of the two mirroring $\theta_{\text{zero strain}}$ directions (see SI). A homogeneous distribution of both properties is achieved, especially when considering the very center of the PDMS substrate, which is the area usually observed during live imaging of cells. Comparing the results obtained from both approaches, a very good agreement between experimental and simulated data is achieved (see table 3). The small difference in the strain modulus values could be due to the difficulties in simulating the exact clamping of PDMS substrate (it has been simplified with respect to the experimental case in the FEM analysis).

## 5. Examples of applications of cell stretching devices

In this section we will consider some examples, from our and other laboratories, of applications of stretching devices in mechanobiology studies.

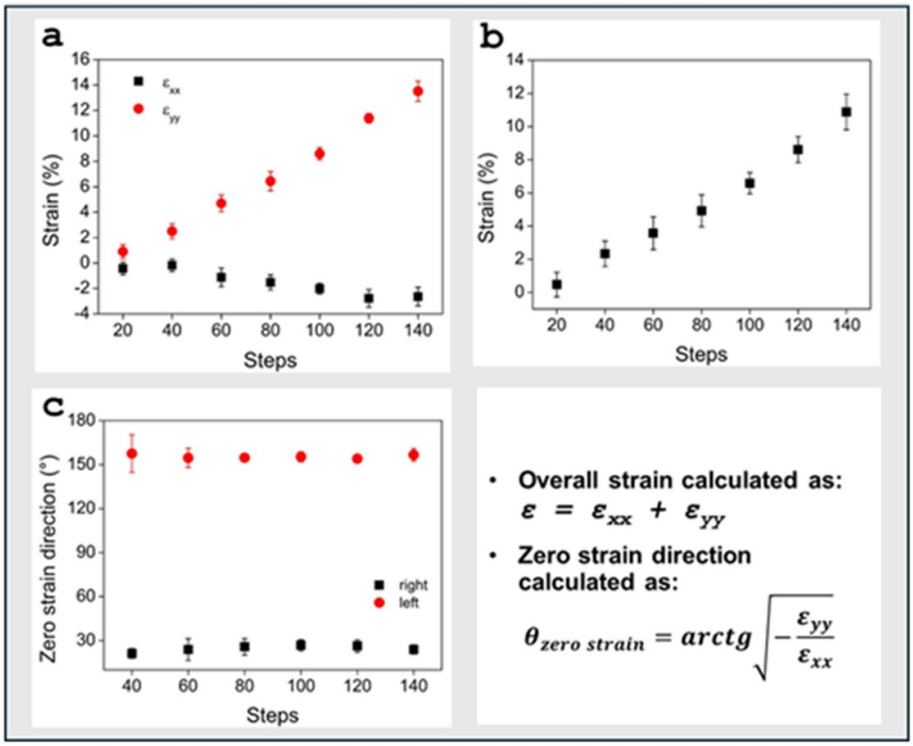


**Figure 9.** (a) Evolution of $\varepsilon_{xx}$ and $\varepsilon_{yy}$ as a function of the increasing number of steps of the stepper motor; (b) overall strain estimation: (c) evaluation of the zero-strain mirror angles for the different stretching conditions.

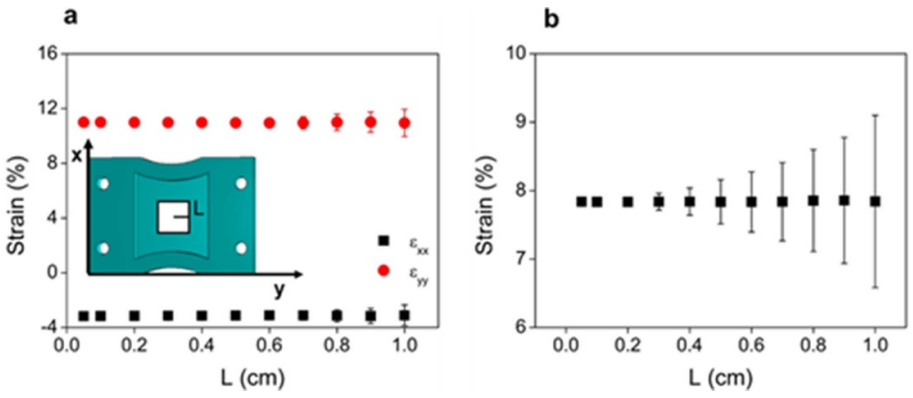


**Figure 10.** Data from the FEM simulation of the uniaxial PDMS substrate as a function of the semi-side value $L$: (a) average $\varepsilon_{xx}$ and $\varepsilon_{yy}$ over the square of side $2L$. In the inset, a small schematic representation of the region of interest of variable area $= (2L)^2$ is reported; (b) the overall value of the strain $\varepsilon$ is reported.

We will concentrate on some of the typical aspects of cell behavior that are affected by stretching, such as the specific orientation of cells with respect to the geometry of the stimulus, the gene and ribonucleic acid (RNA) expression profiles, cell migration and cell proliferation. Both cell monolayers, in which a role is assigned to cadherins at the cell–cell junctions besides the cell/support adhesion provided by integrins [125, 126], and isolated cells can be studied. Here, we will mainly focus on the case of isolated cells. Cell stretching devices are typically exploited to study the behavior of cells residing in tissues physiologically exposed to cyclic stretching. In the previous sections we mentioned experiments regarding cells of the cardiac and vascular systems, but it is worthwhile mentioning also experiments performed on cells of the lung, exposed to cyclic stretching during breathing, and of the gut, exposed to cyclic stretching during peristaltic motion. Experiments involving cells of the lung have concentrated on the effect of cyclic stretching on permeability, cell proliferation, and myofibroblast differentiation of primary lung fibroblasts [127–130]. In the case of cells of the gut, several studies analyzed the effect of cyclic stretching on cell proliferation, differentiation, gene expression and the orientation of cells in a monolayer [131–134]. Many of these studies are currently performed exploiting also organ-on-chip devices [135]. These devices are based on the fabrication of

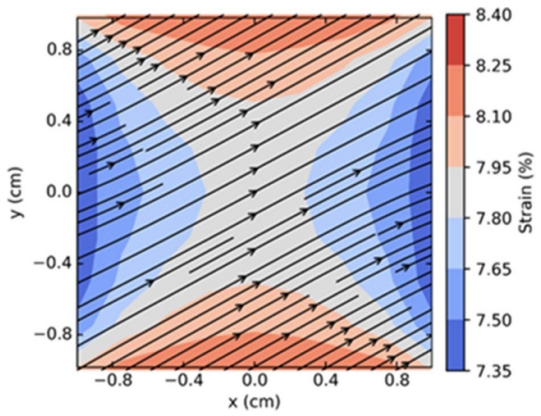


**Figure 11.** Contour map of the overall strain ε overlaid with the zero strain direction (black arrows) obtained from the FEM simulation of the uniaxial PDMS substrate.

**Table 3.** Comparison between relevant data from the experimental calibration (at 100 steps) and the FEM simulation (applying a deformation of 2.04 mm per side).

| | Experimental calibration | FEM simulation |
|---|---|---|
| $E$ | $(6.6 \pm 0.5)\%$ | $(7.84 \pm 0.01)\%$ |
| $\theta_{\text{zero strain}}$ | $(27 \pm 4)^\circ$ and $(155 \pm 4)^\circ$ | $(28.20 \pm 0.02)^\circ$ |

miniaturized microfluidic chips in which the mechanical stretching is typically produced by vacuum actuation and this stimulus can be coupled to other cues such as chemical gradients or shear stress.

### 5.1. Orientation of cells (rotations)

Since the first observations that cells of the smooth muscle of the vessels, not exposed to the pulsating blood flux, are oriented according to a certain angle with respect to the longitudinal axis of the vessel, an interest arose focused on understanding the role of mechanical stretching stimuli on the orientation of cells [14]. In many cases, for different cell types growing on an elastomeric substrate, such as endothelial cells, epithelial cells, fibroblasts, osteoblasts and mesenchymal stem cells, it was found that cells tended to align themselves almost perpendicularly to the main stretching direction [123, 124, 136–140]. Before going deeper into the details of this reorientation process for cells in a 2D environment, it is important to stress that, when cells in a 3D environment of hydrogels have been investigated under the effect of cyclic stretching, a prevalent orientation parallel to the stretching direction has been found [19, 20, 141–143]. This difference could be related to a sort of contact guidance due to the fibers in the ECM that, in turn, are acted upon by the contraction of the embedded cells. The stretch avoidance aspect, which will be discussed below, could also have a reduced effect due to the inability of a 3D soft matrix to transmit all the stretch to cells. Considering the reorientation process for cells on a 2D support, a relevant role is attributed to the cytoskeletal structures and in particular to stress fibers, focal adhesion complexes, microtubules and in general to the mechanical homeostasis of cells [73, 144]. The typical orientation adopted by cells upon cyclic stretching suggested that cells could follow a reorientation process in search of the direction that minimizes the strain deformation on the substrate (zero strain directions) [122, 145]. Considering that most of the experiments were performed with uniaxial stretchers with the two principal directions characterized by strains of opposite signs, two mirror directions of zero strain appeared in the images of the cells after a period of cyclic stretching, as shown by the example in figure 12. According to other models, also the role of focal adhesions is particularly relevant, leading to the detachment from the substrate and the corresponding rotation of the stress fibers, to which the focal adhesion complexes are connected [146]. However, this interpretation neglects the elastic energy of the cell as a 2D system, where an orthotropic model should be considered with two elastic constants in two perpendicular directions. One of the first works suggesting this aspect was that by Livne *et al* [123]. Their theory was able to reproduce the equilibrium orientations of the cells for stretching protocols with different ratios of the strains in the two perpendicular directions. These orientations do not coincide with the direction of zero strain nor with the direction of zero stress of the substrate. The consideration of the elastic energy minimization implies a biological advantage for the reorientation process: cells align in the direction producing the minimum cost for the tissue to produce a cyclic stretching. At the same time, the introduction of an energetic cost of a conformation opens the way to an analysis of the process based on statistical mechanics [147]. In this case, the reorientation dynamics can be seen as a relaxation process

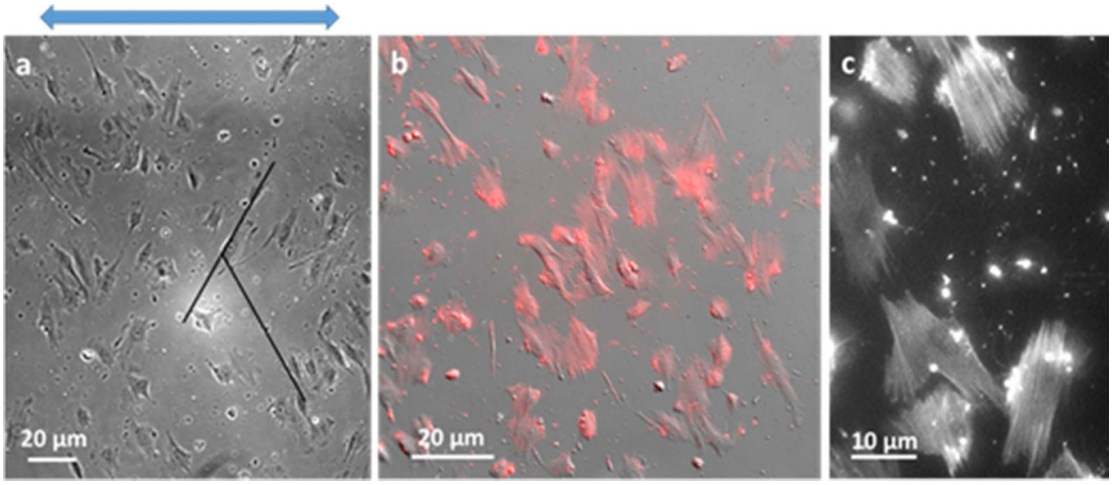


**Figure 12.** Example of cell orientation in the case of uniaxial cyclic stretching. (a) Phase contrast image of WS1 cells on a PDMS support and exposed to a uniaxial cell stretching protocol (1 Hz, 10% max strain, triangular signal for 8 h). The blue arrow shows the direction of stretching whereas the two black lines show the two directions of preferential cell orientation; (b) another region of the same experiment as in (a), but showing an overlap of a DIC image and a fluorescent image in which F-actin has been live stained with SIR-actin (Spyrochrome); (c) fluorescent image in which F-actin has been live stained with SIR-actin (Spyrochrome).

characterized by a dissipative mechanism [148]. Considering the dynamics of the reorientation process, a common result is the fact that a higher strain-rate produces an increase of the speed of the process [124]. Moreover, it has been proposed that cell reorientation starting from an initial condition could follow two different pathways [123, 149, 150]. In particular, cells can start rotating while keeping almost intact the stress fibers apparatus, or they can fluidize and rebuild stress fibers in a direction near to the new equilibrium one. Based on our experiments (see figure 13), we propose that, if cells have been stretched by a non-isotropic stretching stimulus for a time long enough to reach a homeostatic situation, it is very rare to avoid cell rounding if the stretching direction is changed by a large rotation angle (for example 90°). In our case we have no direct evidence of a fluidization event, but we can speculate, considering previous experiment in the literature [27, 151], that this process is probably occurring. Achieving the homeostatic condition for cells subjected to cyclic stretching typically involves their elongation in a direction almost perpendicular to the direction of stretching. In this situation, by changing the stretching direction, stress fibers can be destroyed [151] and the nucleus can decouple from cytoskeletal elements [56] and new stress fibers form with a direction near to the new equilibrium condition. In the case of cells with an initial orientation not too far from their equilibrium direction, a rotation of the stress fibers mainly due to unbinding and immediate re-binding of integrins can occur .

### 5.2. Cell proliferation and migration in the presence of cyclic stretching

Another common effect of cyclic stretching stimulus is the increase of cell proliferation [152, 153]. Several investigations tried to understand the molecular mechanisms ruling this effect. Among the different explanations, it has been proposed that the increased permeability of cells to $Ca^{2+}$ due to the action of the stretching process on mechanosensitive channels, specifically on Piezo 1 channels for epithelial cells, could activate the ERK1/2-MEK1/2 pathway inducing transcription of cyclin B which finally promotes mitosis [68]. The effect of cyclic stretching has been largely studied in the case of skeletal muscle cells for the physiological relevance of this process for the tissue they form [154, 155]. In many cases, primary stem cells of these tissues are considered quiescent cells unless stimulated by mechanical forces. Mechanical stimuli are able to activate these cells to assure the conservation of the pool of stem cells but at the same time, the production of committed cells, such as myoblasts, is also increased. In many cases, using *in-vitro* cyclic stretching experiments allowed acquiring a lot of information about how both stem cells and myoblasts are affected by mechanical stimuli [156]. Cells re-enter the cell cycle as a consequence of growth factors released from the ECM upon stretching, activation of metalloproteinases and the increased influx of calcium promoting the calmodulin pathway or the activation of other biochemical pathways [157]. Interestingly, in the case of C2C12 myoblasts, it has been found that the mechanical stretching, depending on its specific parameters such as duration, magnitude and frequency, could induce both proliferation and apoptosis [158, 159]. Another mechanism that is affected by cyclic stretching, leading to increased cell proliferation, is related to the Hippo pathway. In this case, cyclic stretching is responsible for an increased accumulation of YAP/TAZ in the nucleus (figure 14). This mechanism has been demonstrated in the case of mouse embryonic

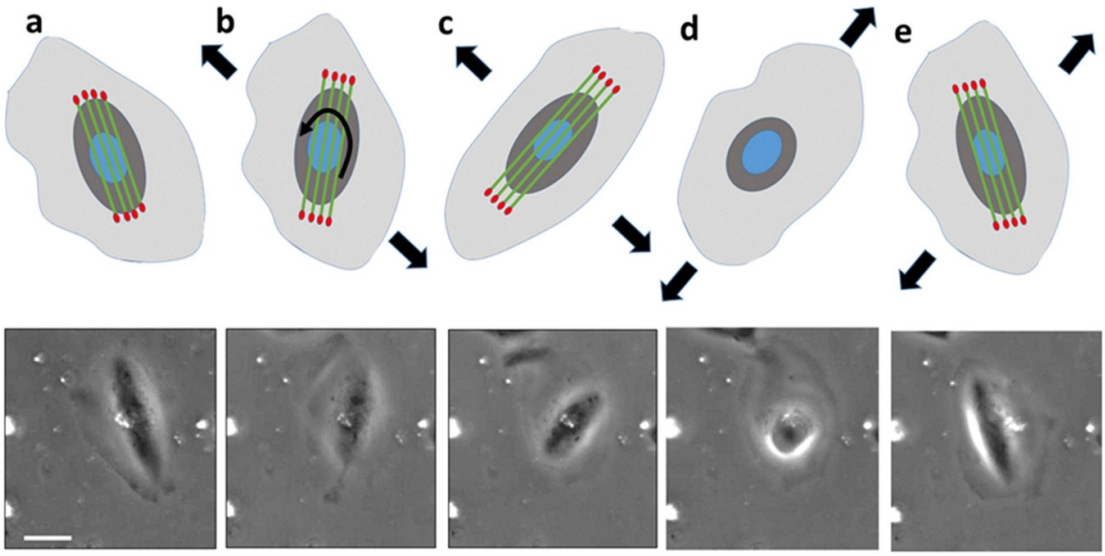


**Figure 13.** Reorientation process for a human cardiac fibroblast (HCF) cell. (a) initial configuration of the cell before the stretching stimulus is applied; (b) the application of a stretching stimulus in the direction shown by the black arrows produces a rotation of the cell apparently maintaining the central structure and the stress fibers. The curved black arrow shows a visible (in the movie of the process—see movie S3) rotation of the cell cytoplasm around the nucleus in a direction opposite to the rotation of the central part of the cell. (c) the equilibrium direction of the cell with respect to the uniaxial strain is reached by the cell; (d) changing the stretching direction by 90° the central body of the cell gets round; (e) continuing the stretching protocol produces the elongation of the stress fibers in the new equilibrium direction.

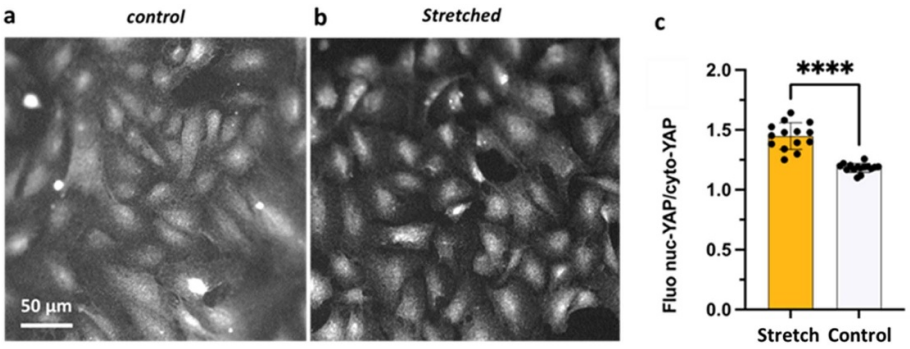


**Figure 14.** Immunofluorescence analysis of yes associated protein (YAP) of HCF cells showing the partitioning between cytoplasm and the nucleus. For control cells (a) on a PDMS substrate in static conditions, the difference between YAP partitioning in the nucleus and the cytoplasm is much lower than in the case of the same type of cells on PDMS exposed to cyclic stretch (b) (1 Hz, 10% strain). (c) The YAP partitioning has been evaluated by considering the ratio of the fluorescence intensity inside a small square (with a side of a few pixels) positioned in the nuclear region, identified by DAPI staining, or in the cytoplasmic region.

fibroblasts, for which an increase of the proliferation has been observed when cells were growing on soft substrates and subjected to cyclic stretching [22]. This example once again clarifies that exposing cells to cyclic stretching is equivalent to letting them grow on a stiffer substrate, i.e. that mechanical signals coming from inside produce the same effect of mechanical signals from outside the cell.

Cell migration is a fundamental process in cell biology for the development of normal tissues and organs, as well as for several pathological processes, and it can be affected by the presence of a cyclic mechanical stretching signal both in the case of single-cell migration and in the case of collective cell migration such as in wound closure processes [160–166]. Considering that cell migration is a process requiring, at least in the mesenchymal type of migration, adhesion with the substrate through integrins and focal adhesion complexes and that cyclic stretching affects the organization and dynamics of these cell components, it is clear that cyclic stretching can affect cell migration. Moreover, we already considered that cyclic stretching produces an orientation of cells mainly associated with an orientation of stress fibers. Cell migration requires polarization of cells (in this case polarization is similar but not equal to orientation, because in the first case there is an asymmetry between the rear and the front portions of the cell in terms of internal cytoskeleton organization, whereas orientation does not distinguish between a rear and front part of the cell) and cyclic stretching favors a specific orientation in a direction almost perpendicular to the stretching direction. Accordingly, the

direction of migration corresponding to the direction of stress fibers and perpendicular to the stretching direction could be favored. Asymmetric cell migration has been for example observed for *Dictyostelium* cells, where they preferentially migrate along a direction perpendicular to the stretching direction [164]. We recently demonstrated that the same is true for Balb-3T3 cells [30]. In figure S19 a comparison of two rose plots obtained for cells in a static condition and on a substrate undergoing cyclic stretching with 10% amplitude, 1 Hz frequency and a triangular signal, is reported. The asymmetry of the rose plot for stretched cells highlights that migration in the direction perpendicular to the stretching axis is favored. We also observed that migration speed increases for stretched cells and the persistence time decreases. At the same time, we observed that for cells that do not significantly express stress fibers, as in the case of glioma cells, and whose migration is independent of actin filaments, cyclic stretching is not able to induce a specific cell orientation and no effect of stretching is observed in the process of migration. Interestingly, for fish keratocytes it has been found that cells exposed to cyclic stretching preferentially migrate in the same direction of stretching [163]. This behavior could be explained by considering that, for this type of cells, migration typically occurs in the direction perpendicular to the stress fibers in the cell body. Based on these pieces of evidence, the alignment of stress fibers by cyclic stretching can thus affect the migration direction. From a physiological point of view, the effect of cyclic stretching on collective cell migration is relevant in the case of organs and tissues growth and development. The typical *in-vitro* experiment to analyze collective cell migration is that of wound healing. In this specific case two cell fronts are created by removing a thin line of cells that formed a complete monolayer. The presence of a cyclic stretching stimulus during the healing phase, in which cells migrate to close the wound, has been demonstrated to act in a biphasic way with the stretching amplitude [160]. A very small amplitude of oscillation is able to decrease the healing time, whereas an intermediate stretching amplitude produces a slower wound closure time. The closure mechanism shows an acceleration perpendicularly to the stretching direction, producing a different closure mechanism compared to the static mode. In line with what stated above, the faster closure process could also be related to an increase of cell proliferation. A further increase of the stretching amplitude decreases the closure time again. In general, the study of the effect of stretching on the healing processes could be relevant for regenerative medicine and healthcare [167].

## 6. Integration of TFM with cell stretching devices

In the context of mechanobiology, the technique of TFM allowed a breakthrough in the understanding of the force applied by cells on their environment [32, 168]. Nowadays both 2D and 3D TFM can be performed but, to the best of our knowledge, only the 2D version has been coupled to stretching devices. A prerequisite for implementing TFM in a stretching device is to have a soft hydrogel or elastomer layer firmly connected to the elastomer by which the stretching is performed [27]. As mentioned above, the transmission of the mechanical strain to the soft layer has to be verified. In general, in the case of a PDMS stretching support, the deposition of another softer PDMS layer such as that obtained from PDMS 527 [117] assures a good attachment, but the deposition of a PAA layer, the hydrogel of choice for TFM experiments, requires specific chemical steps to prepare the surfaces [29]. In any case, when using a hydrogel undergoing a stretching deformation, care should be taken about the hydraulic pressure resulting from water exiting from the surface and acting on the above residing cells [97].

The main goal of TFM implemented in cell stretcher devices is that of investigating the mechano-adaptive process of cells exposed to an instantaneous stretching event [169]. This process could be relevant for example in cases of vascular remodeling as a consequence of a damage, where cells rapidly undergo a change in their mechanical environment and they have to adapt to the new situation looking for a homeostatic reaction. In the case of human alveolar epithelial cells it has been shown that, upon a step in the stretching of the substrate on which they are growing, the traction force rapidly increases with respect to their initial value [27]. It is to be remarked that the traction force was measured in the stretched condition for cells, where the possible change of the Young modulus of the support, with respect to the relaxed condition, has to be taken into account if the mechanical properties of the support are not linear and a strain-stiffening process occurs. However, when the substrate was taken back to the initial condition, the traction force of the same cells sensibly decreased with respect to the initial stationary condition, followed by a slow increase up to the reference value. This behavior is strongly dependent on the amplitude of the stretching step, and the initial increase of the stress applied by cells on the support after a larger stretching step has a lower ratio of stress increase compared to the initial condition. These results point to a fluidification of the cell cytoskeleton upon a fast cell stretching step with a slow re-equilibration when the stretching stimulus is removed. The same behavior could be the biological reason for cell alignment in the presence of a cyclic stretching stimulus. Indeed, cells could realign in order to reach the tensional homeostatic situation regarding the stress applied to the environment [151]. About this, it has been observed

that, at the onset of cyclic stretching, human umbilical vein endothelial cells fluidize and the stress applied by cells immediately decreases, followed by a slow increase of the tensional stress, but with cells oriented almost perpendicularly to the stretching direction [151]. At the same time, as cells reoriented, the fluctuations of the stress applied to the substrate decreased. These investigations are relevant to understand the physiological activity of tissues exposed to cyclic stretching *in-vivo*, in which tensional homeostasis is thought to protect against diseases such as atherosclerosis and fibrosis.

## 7. Conclusions and future directions

In this work we considered how cell stretcher devices can provide a fundamental means to answer some of the most compelling questions about mechanobiology. These devices allow reproducing *in-vitro* important stimuli that cells typically experience *in-vivo*, making the conditions for cell cultures increasingly closer to the physiological ones. One of the most important aspects of this technique is the possibility of observing in real-time the reaction of cells to mechanical stimuli [170] and to variations of the amplitude, frequency and direction of the stimuli. In fact, it is nowadays emerging that cells are able to transduce different mechanical stimuli in different biochemical pathways and understanding the details of these processes could improve many aspects of regenerative medicine and therapies based not only on chemical but also on physical means. The use of a device where cells are challenged just by mechanical stimuli oversimplifies the real physiological situation where other stimuli, such as chemical diffusion of paracrine molecules, shear stress or interstitial fluid flow might be present. So it would be important to consider experiments at tissue level, where different types of cells, such as for example cardiac fibroblasts and cardiomyocytes, are exposed to different stimuli [171, 172]. If seeing is believing, the integration of live imaging based on high time and spatial resolutions with cell stretchers further improves the potentiality of these approaches. In light of this, we focused on the construction and description of devices coupled to optical systems. In many cases, the vast majority of currently used devices coupled to optical systems allow imaging of the cells just in static conditions, when the cyclic process is stopped. This is mainly due to the vertical translation of the substrate on which cells are growing during the stretching protocol, which hinders the maintenance of the *in-focus* condition. Developments towards the implementation of devices with an almost vertically immobile surface during the stretching protocol would add an increased time resolution to the technique to follow very fast reactions of cells to mechanical stimuli. From the technical point of view, it would also be extremely important to develop a *z-follower* mechanism to continuously have the sample surface in-focus, given that a small change of this vertical position seems unavoidable due to the Poisson effect of elastomers. Moreover, it would be important to couple spinning disc confocal microscopes to cell stretchers. In fact, this type of microscopy allows fast imaging with very low bleaching effects when live fluorescent markers are used. Even if we analyzed just examples of the application of cell stretching devices in the case of 2D cell cultures, we mentioned that the technique has already been implemented with more physiological 3D cultures [170]. However, in this case coupling the stretcher with time-lapse imaging poses even more challenges. For reproducing 'quasi' 3D cell migration, the exploitation of surfaces integrating micropillars produced by photolithography has been pursued [173]. A similar approach can be implemented for cell stretchers. By integrating elastomeric micropillars in the substrates for cell stretchers it could be possible to analyze in a much easier way the invasion of cells in a geometrically changing environment [174]. It could be, for example, possible to study in real-time the change of the invasion mechanism, mesenchymal *vs* amoeboid, when the geometrical confinement is changed. Another aspect on which future developments are highly desirable is the improvements of high throughput systems [98, 175, 176] possibly integrated with full automation and real time imaging. These systems will allow better screening of possible drugs and the identification of their targets.

## Data availability statement

The data cannot be made publicly available upon publication because they are not available in a format that is sufficiently accessible or reusable by other researchers. The data that support the findings of this study are available upon reasonable request from the authors.

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## Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. M B, L C, C V, A A performed conceptualization, methodology; B B, G R, A G, A M, C S, C Z, M M, E O, C C, R T performed experiments, software development and data analysis

## ORCID iDs

Chiara Scagliarini https://orcid.org/0009-0001-7934-1873
Chiara Zannini https://orcid.org/0000-0003-3925-4844
Michele Bianchi https://orcid.org/0000-0002-9660-9894
Andrea Alessandrini https://orcid.org/0000-0002-4782-2365

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