A device for simultaneous live cell imaging during uni-axial mechanical strain or compression

Axel Gerstmair, Giorgio Fois, Siegfried Innerbichler, Paul Dietl, and Edward Felder

Institute for General Physiology, University of Ulm, Ulm, Germany; and Innerbichler GmbH, Breitenbach am Inn, Austria

Submitted 9 January 2009; accepted in final form 28 May 2009

Gerstmair A, Fois G, Innerbichler S, Dietl P, Felder E. A device for simultaneous live cell imaging during uni-axial mechanical strain or compression. J Appl Physiol 107: 613–620, 2009. First published June 4, 2009; doi:10.1152/japplphysiol.00012.2009.—Mechanical stimuli control multiple cellular processes such as secretion, growth, and differentiation. A widely used method to investigate cell strain ex vivo is stretching an elastic membrane to which cells adhere. However, simultaneous imaging of dynamic signals from single living cells grown on elastic substrates during uni-axial changes of cell length is usually hampered by the movement of the sample along the strain axis out of the narrow optical field of view. We used a thin, prestrained, elastic chamber as growth substrate for the cells and deformed the chamber with a computer-controlled stretch device. An algorithm that compensates the lateral displacement during stretch kept any selected point of the whole chamber at a constant position on the microscope during strain or relaxation (compression). Adherent cells or other materials that adhere to the bottom of the chamber at any given position could be imaged during controlled positive (stretch) or negative (compression) changes of cell length. The system was tested on living alveolar type II cells, in which mechanical effects on secretion have been intensively investigated in the past.

mechanotransduction; motion compensation; elastic membrane; alveolar

IN VIVO, A MULTITUDE OF CELLS are subject to different types of deformation. Equi-biaxial strain is predicted for tissues such as the pulmonary alveolus or the urinary bladder where a sphere increases its volume, whereas unidirectional strain occurs when a cylinder expands, such as in blood vessels after increase of the luminal pressure. Identifying mechanosensing molecules and how they transduce a mechanical load to a biological signal (ion current, enzyme activation, etc.) are among the most intriguing and challenging issues in current biology (4, 9, 12). Therefore, imaging of living cells subject to various types of mechanical stimulation is becoming an indispensable tool to investigate mechanobiology and mechanotransduction on the level of single molecules (14). A multitude of devices and systems for mechanical stretching of tissues or cells ex vivo has been developed and is reviewed in detail by others (2, 3).

Tissue deformation is typically transferred to mechanosensitive molecules by the cytoskeleton, which is anchored to the extracellular matrix by integrin-containing adhesion structures such as focal adhesions (reviewed in Ref. 10). Hence a common approach to study the effects of mechanical stimulation is to apply tension to the growth substrate, typically by stretching elastomeric membranes on which the cells have been cultivated (2, 3). Although most studies have been focusing on tensile cell strain, these systems could in principle also be used to inves-
between both and providing the necessary counterforce to keep the metal band under tension. Any displacement of the first block leads to a displacement of the other block for the same amount but in the opposite direction (see arrows in Fig. 1A). An increase of the distance between the two blocks requires the active force of the motor, whereas a decrease follows the passive, attractive force of the spring.

The compensation component is comprised of the carrier plate on top of a linear motion guide (not shown in Fig. 1A) and the compensation motor that can laterally displace the carrier plate via a ball screw. As all parts of the deforming component are fixed to the carrier plate, this component is moved as a whole by the compensation motor.

The two DC servo motors for the deformation and the compensation component (both: model 3056K 024B, Faulhaber, Schönai, Germany) are both connected to a motion control unit (MCBL 2805, Faulhaber), which in turn is controlled by a computer using a custom-written software. The main function of the software is to compensate lateral movement during stretch or compression by moving the carrier plate with the deformation component. In addition, the graphical user interface of the software allows the user to do the following.

1) To precisely define any desired strain protocol, including cyclic protocols with different characteristics (linear, sinusoidal, sigmoidal) and the option for inserting pauses or loops to repeat a specific sequence.

2) To control the position of the membrane chamber along the stretch axis independently from movements for motion compensation including the option to store positions for a later observation.

3) To freely choose the distance between the two sliding blocks of the SC component to mount membrane chambers of different lengths. Thus membrane chambers can be mounted with any degree of prestrain (see Membrane chamber including prestrain holder) that had been applied prior to mounting onto the membrane holder.

---

**Fig. 1. Schematic of the strain/compression (SC) device.**

**A:** a sketch of the SC device mounted on the microscope with the membrane chamber attached. The carrier plate is shown in gray with all attached components in matching colors. The SC motor changes the distance between the sliding blocks via a ball screw and a metal band (see Technical Description of Entire Device) and deforms the mounted membrane chamber. The entire carrier plate can be moved laterally with the ball screw driven by the compensation motor (both in blue). The red arrows indicate movements during deformation (stretch as solid arrows and compression as dashed arrows) and the compensatory movement of the carrier plate. B: the membrane chamber with the membrane shaped into a tray by 2 blocks. In the side view (top right) one of the blocks is shown before screwing it together and reveals how the tray is formed. The two double hooks of the prestrain holder (B, bottom), onto which the membrane chamber is mounted, can be moved freely along the plate rail and fastened at any position. The observation window allows microscopic inspection of the cells during cultivation of the cells without removing the prestrain holder from the protective culture dish. C: both parts readily assembled to be used for cell culture. D: a photograph of the device mounted onto an inverted microscope. The objective of the microscope is visible underneath the membrane chamber.
Membrane chamber including prestrain holder. The membrane chamber that will eventually be mounted to the sliding blocks of the SC component consists of the transparent membrane (thickness 127 μm; Specialty Manufacturing, Saginaw, MI) and two membrane holders, which clamp both ends of the membrane in such a way that the membrane assumes a shape with a flat bottom and two side walls (angle of ~60°). Thereby, membrane holders and membrane form a tray, which prevents leakage of bath solution. Figure 1B, top right, shows a side view of the membrane chamber. One of the two membrane holders is not clamping the membrane yet. Prior to the actual deformation on the SC device the membrane chamber is hooked onto a prestrain holder where it can remain as long as necessary, typically during the entire procedure where the membrane is treated for cell culture and, if necessary, also during incubation in the CO2 incubator for several days. Metal rods in the membrane holders allow for the mounting of the membrane chamber onto the two sliding blocks of the SC component. To avoid movement or perturbation of the membrane, the chamber can be easily removed from the prestrain holder. The design of the prestrain holder and the sliding blocks guarantees that none of the two membrane holders is rotated during this transfer. The distance of the two membrane holders can be adjusted freely in the prestrain holder by sliding them along a plate rail (see Fig. 1B) and fixing them at any desired position with a locking screw.

To create a flat bottom of the membrane and especially also to allow a compression of the cells, the membrane chambers are always mounted with a certain degree of prestrain before seeding the cells. The amount of prestrain is theoretically limited by the total strain that can mechanically be applied to a membrane (~100%). For our experiments, a prestrain of 30% was chosen. If cells have to be compressed, the amount of prestrain has to be high enough so that the membrane still remains under tension after relaxation. A remaining stretch of 15% is sufficient for that purpose.

Algorithm for Motion Compensation

The algorithm for motion compensation is entirely based on the distance of the observed area from the center of the membrane. To keep a certain part of the membrane in the field of view of the microscope during stretch or compression of the cells, it is necessary to develop a model describing the deformation of the membrane. Using such a model, it is possible to predict the movement of a point anywhere on the membrane during membrane deformation and thus to counteract its lateral displacement by moving the compensation component (see also Fig. 2). As our uni-directional stretch device displays a homogenous strain field, there is a simple linear correlation between the distance x0 of an observed point from the center and the displacement Δx of the point when the membrane changes its length. That is, at a point at the very center of the membrane does not move (Δx = 0), but with increasing distance from the center the movement becomes larger when the membrane is deformed. Let x be the relative amount of strain applied to the membrane (σ > 0 for stretch; σ < 0 for contraction) then

\[ \Delta x = \sigma x_0 \] (1)

Thus the new position x\text{deform} of the observed point can simply be obtained as

\[ x_{\text{deform}} = x_0 + \Delta x \] (2)

Provided the membrane is in its initial state, Equations 1 and 2 suffice to determine the amount of movement and the new position of any observed point. However, if the membrane has already been stretched or contracted, the applied deformation must be considered when calculating Δx. At first, x0 must be eliminated from Equation 2 by solving Equation 1 for x0 and substituting it in Equation 2, which gives

\[ x_{\text{deform}} = \frac{\Delta x}{\sigma + 1} + \Delta x = \left(\frac{1}{\sigma + 1}\right)\Delta x = \left(1 + \frac{1}{\sigma}\right)\Delta x \] (3)

Solving for Δx finally gives

\[ \Delta x = \left(\frac{\sigma}{\sigma + 1}\right)x_{\text{deform}} \] (4)

Using Equations 1, 2, and 4, it is possible to determine the displacement under strain, compression, and even between different levels of deformation.

Calibration of SC Device Before the Experiment

Prior to the experiment it is essential to position the base plate of the SC device so that the center of the membrane chamber is aligned with the optical axis. This is done by moving the entire SC device with the stage of the microscope. Moreover, it is necessary to provide the software with the precise distance between the two sliding blocks at a given reference position. Both can be accomplished by inserting a membrane chamber dummy made of solid metal into the sliding blocks. The dummy has a drilling in the center for alignment, and the reference distance between the two sliding blocks is naturally defined by the width of the metal piece. After this calibration, the stage of the microscope may only be used for movements perpendicular to the stretch axis, whereas movements along the stretch axis have to be performed by moving the carrier plate via the SC software (cursor keys on controlling computer).

Treatment of the Elastic Membranes for Cell Culture

The elastic membrane was cut into a rectangular piece (9×2 cm) and clamped into the membrane holders that shape the membrane into a chamber (see Stretch/compression device). Then the assembled membrane chamber was hooked onto the prestrain holder, thereby stretching the membrane chamber to ~130% of its relaxed length. This prestretch remained unchanged during all following steps (sterilization, coating of the membrane, seeding, and cultivation of cells) until the membrane chamber was mounted onto the SC device. A cleaning step was performed before each experiment to remove dirt and, if the membrane holder had been used in a previous experiment, cell debris. Each membrane chamber was filled with 70% isopropanol and carefully wiped clean and dried with Q-tips several times. Next, distilled water was repeatedly added and removed with a vacuum pump. Finally, the chambers were either wrapped in aluminum foil.

Fig. 2. Strain model. In the upper, unstretched membrane 2 positions are marked: the center and a position 10 mm distant from the center (≈ x0). The lower membrane chamber is stretched by 20%. The point in the center does not move under strain, whereas the second point is moved by 2 mm (≈ Δx) away from the center. This is caused by the length increase of the membrane that is assumed to be distributed equally. Obviously, this interrelationship is true for every point on the membrane and, therefore, provides the basis for the motion compensation algorithm.
and autoclaved or treated with ultraviolet light (10 min at 100 mJ/cm²). To facilitate the adherence of alveolar type II (ATII) cells, the silastic membranes of the membrane chambers were coated with fibronectin by filling the chambers with 750 μl of a fibronectin solution (Sigma, St. Louis; 5 μg/ml in phosphate buffered saline) and incubating the chambers for at least 4 h at 4°C.

Cell Isolation and Preparation for the Experiment

All experimental protocols were approved by the Regierungspraesidium Tuebingen/Baden-Wurtemberg/Germany (TVN 833) prior to conducting the experiments. ATII cells were isolated from male Sprague-Dawley rats with a body weight of 180 to 200 g according to the method by Dobbs (5) with modifications, as described previously (8). Briefly, rats were anesthetized (ketamin 10% and xylazil 2%) and injected with heparin (400 IU/kg). Lungs were perfused, removed, washed, and incubated twice with elastase and trypsin at 37°C for 15 min, immered in DNase-containing solution, and sliced into bits of ~1 mm³. Enzyme reaction was stopped by incubation with FCS (37°C, 2 min). The tissue was then filtered three times through gauze and nylon meshes (mesh width: 150, 20, and 10 μm) and the final filtrate was centrifuged for 8 min at 130 g. After suspending in DMEM, the cells were put on IgG-coated plastic dishes and incubated at 37°C for 15 min. Non-adherent cells were centrifuged for 8 min at 130 g, suspended in DMEM with 10% FCS and 1% penicillin/streptomycin (10,000 U/ml penicillin, 10 mg/ml streptomycin), and seeded onto the fibronectin-coated membrane chamber (300 μl cell suspension plus 700 μl medium), which had been mounted onto the prestrain holder. Both were then transferred into a regular 10 cm dish for cell culture and were cultivated at 37°C/5% CO₂ for 2 days.

Microscopic Analysis of Physical/Technical Parameters

The SC device was screwed onto the motorized stage (Prior, ProScan II) of an inverted Zeiss Cell Observer microscope. Before each experiment, the system was calibrated as described above to enable accurate compensation of the displacement during stretch. The cells, which had been cultivated in the prestrain holder for 2 days, were stained with either 4 μM Fura-2 or with 0.5–1 μM calcein-AM (both: Molecular Probes) by 20 min preincubation at 37°C/5% CO₂ in medium. Calcein stains the cytoplasm and leaks from cells into the bath solution when the membrane barrier is impaired. Fura-2 is a Ca²⁺ dye, where a sequential excitation with two different wavelength allows a ratiometric and therefore highly reliable measurement of the intracellular Ca²⁺ concentration. For both experiments, the medium in the membrane chamber was replaced with bath solution (in mM: 140 NaCl, 5 KCl, 1 MgCl₂, 2 CaCl₂, 5 glucose, 10 HEPES, pH 7.4). Finally, the membrane was mounted onto the SC device. For Fig. 5, one single stretch of 20% was applied within 1 s while continuously imaging the cells with a ×40 Zeiss Fluar oil immersion objective before, during, and after having applied and maintaining the stretch (see also Fig. 5 A). A region 2 mm distant from the center point of the membrane measured parallel to the stretch axis and 1 mm distant perpendicular from it was chosen. Using a 490- to 530-nm emission filter and a sequential dual band illumination with 340 and 380 nm, image pairs with 340 nm and then 380 nm excitation (bandwidth 15 nm for both) were acquired at an acquisition rate of 2.5 image pairs/s. A CoolSnap EZ CCD camera (binning factor 3) with Metamorph software was used for image acquisition and to control a polychromator (Visi-Chrome). The image pairs of each time point were then digitally aligned (ImageJ, Plug-in: image stabilizer), the background was subtracted, and the ratiometric image was calculated with ImageJ software.

For Fig. 6, the cells were stretched to 60%, then the stretch was held for 10 s followed by relaxation of the membrane chamber that was stopped when the length of the membrane chamber was reduced by 15% compared with its initial length (see Fig. 6 A). The stretch/compression rate was 1%/s. Pictures were taken at the start and end point and at 60% stretch under static conditions (phase contrast and fluorescence) as well as during the actual stretch and compression phase (fluorescence only; see Fig. 6, B and C) at an acquisition rate of 3 images/s. Cells were illuminated at 480 nm and the emission was recorded at 510 nm. A ×20 Zeiss plan-apochromat objective was used. Images were acquired with a CoolSnap EZ CCD camera (no binning) and Metamorph software.

Statistical Analysis

Unless noted otherwise, all results are shown as means ± SE. Student’s t-test (paired and unpaired as noted) was applied to test for differences in the experimental groups and a value of P < 0.05 was considered significant.
RESULTS

Testing the SC Device

Benchmarks of the SC device. The size of the area in the membrane chamber, where cells can be cultivated, is 12 mm × ~20 mm. As mentioned above, the latter can be adjusted by moving the two membrane holders on the plate rail of the prestrain holder. The total maximum stretch that can be applied is ~100% including the prestrain, which in turn will vary if cells have to be compressed (see also MATERIALS AND METHODS).

Fig. 4. Motion compensation. A: example of the displacement of the region of interest without motion compensation. A membrane stained with ink was moved 2,950 μm away from the center of the membrane chamber and then incrementally stretched. The labels on top indicate the amount of strain. Already at 6% stretch most of the original area of interest has moved out of the field of view. B: using motion compensation, the field of view remains almost constant. C: the stained membrane was stretched up to 50% with motion compensation, then relaxed to the initial values and the mean relative displacement ± SE of 5 different positions (2,000 μm to 3,575 μm distant from the center of the membrane chamber) is shown.

Fig. 5. Image acquisition of ATH cells subject to one fast single stretch with motion compensation applied. A: cells were continuously imaged for 53.6 s. After 4 s a 20% stretch with a duration of 1 s was applied and 4.6 s after the end of the stretch the images were again in focus. B: examples of the continuously acquired image sequence (see corresponding numbers in A) showing ratiometric Fura-2 images, calculated from 2 consecutive images with different excitation wavelengths (false color code: blue-red-yellow-white indicate Ca^{2+} concentrations from low to high). A ×40 oil-immersion objective was used and the position of the objective was off the center point of the membrane parallel as well as perpendicular to the stretch axis. The latter explains the vertical shift of the image between panels 1 and 2. C: the time course of the intracellular Ca^{2+} concentration demonstrates a slightly different response to the mechanical stimulus of the 4 cells indicated in B.
Fig. 6. Image acquisition of ATII cells during continuous stretch with motion compensation applied. A: cells were stretched to 60%, relaxed, and then compressed to −15%. The numbers in circles and the width of the arrow correspond to the time points in the following panels. B: 4 images from the image sequence that was automatically acquired during the entire stretch protocol (125 s) with a ×20 air objective. Cells are labeled with the cytoplasmic dye Calcein. The same cell has been marked with an arrow in all 4 images to show the displacement of the cell during stretch and demonstrates shortcomings of the lateral motion compensation. C: image details from the region that is marked with a white frame in B. In all images precisely the same region was extracted from the original image sequence. The series of 5 consecutive frames (and another frame 7 s later) demonstrates that image quality is sufficient to perform image analysis on a cellular level at high temporal resolution. The diagram shows the decrease of the overall calcein fluorescence signal of a mechanically injured cell (cell 2) and an uninjured cell (cell 1). Note that cell organelles (single lamellar bodies) can clearly be distinguished by the lack of fluorescence.

The maximum strain rate is 40%/s. The maximum values for cyclic stretch are 4 Hz for 10% stretch, 1 Hz for 20% stretch, and 1 Hz for 30% stretch.

Precision of carrier plate movement. The precision of the carrier plate movement was tested by changing its position in consecutive steps of 1, 10, and 100 μm, respectively, and measuring the displacement of fluorescent beads in the image. We found that the correlation of the nominal value and the actual displacement is strictly linear with \( P < 0.001 \) in all step sizes \( (n = 3; 2\text{-tailed} \, t\text{-test}) \). The actual movement of each single step deviated from the nominal movement by \( -8.2 \pm 2.5\% \) (mean ± SE) for 1-μm steps, \( +3.1 \pm 0.1\% \) for 10-μm steps, and \( +3.0 \pm 0.1\% \) for 100-μm steps. At step size 1 μm, the smallest step size tested, the first one or two steps did not reach the nominal step size and the actual step size increased minimally toward the end of the test run, indicating a small mechanical tolerance.

Membrane compression perpendicular to the stretch axis. A series of three tests was conducted to analyze the degree of compression perpendicular to the strain axis when the membrane is stretched. That is, at the center of the membrane (\( \pm 0 \) μm) and at 1,000 μm and 3,000 μm distance from center \( (n = 3 \) for each distance). As shown in Fig. 3, the lateral position on the membrane affects the amount of compression. The maximum compression of 16.58% was measured at the center. The compression gradually decreased when approaching the region closer to the membrane holders, where the membrane is clamped. We also found that the compression does not increase linearly with strain applied, but increases less at higher strain levels.

Effectiveness of motion compensation. As shown in Fig. 4, A and B, where the same region is depicted with and without motion compensation, the motion compensation successfully counteracts the lateral displacement caused by the stretch. Figure 4C shows the mean relative displacement of five points at different distances from the center and is presented as percentage of that distance. The relative displacement remains almost constant with applied strain and does not differ at the different positions. Naturally the absolute values for the displacement (not shown) therefore increase with increasing distance from the center and the motion compensation is most accurate in the center portions of the membrane.

Live Cell Imaging of Alveolar Type II Cells

Before and after applying a single, fast stretch to challenge hardware, software (and the operator) we combined several exacerbating experimental conditions (MATERIALS AND METHODS). In Fig. 5A the blue part of the line that describes the stretch protocol, and the gap in Fig. 5C demonstrates that it took 5.6 s to recapture the same group of cells. This delay was not caused by lagging compensation but by the need to refocus the cells. As the 5.6 s also included the duration of the stretch (1 s) it required 4.6 s to manually refocus the image at a sufficient quality for a ratiometric data analysis of two consecutive images. All images of the time series were available for analysis, of which four are shown in Fig. 5B, panels 1–4. Due to membrane compression and the position of the objective, which was off the centerline by 1 mm, Fig. 5B, panels 2–4, are shifted vertically compared with Fig. 5B, panel 1. The time...
course of the intracellular Ca\(^{2+}\) concentration of four cells is shown in Fig. 5C and demonstrates that cells responded differently to the mechanical stimulus. Cell 3 reached a maximum concentration 12 s after starting the acquisition, whereas the others were recaptured when the Ca\(^{2+}\) levels had already reached a plateau phase.

During continuous strain or compression to demonstrate the applicability of the SC device during an ongoing change of the cell length, we labeled ATII cells with the cytoplasmic dye calcein. It clearly maps the cells with their borders and is used as an indicator for cell damage. Figure 6 displays a time series of the same group of cells during continuous changes of cell length. At a strain amplitude of up to 60% and a stretch rate of 1%/s, only one of several cells lost its fluorescence intensity, indicating that the loss of cell membrane barrier function is very heterogeneous within a cell population grown under the same conditions. The same membrane chamber has also been compressed by 15%, which was not accompanied by apparent changes in cell integrity.

**DISCUSSION**

Visualizing the response of cultivated cells to a mechanical stimulus has become an important tool to investigate various aspects related to mechanotransduction. The number and versatility of microscopic techniques, especially fluorescence detection methods, is steadily increasing, and more and more questions can be addressed with the appropriate fluorescent label. Examples are Ca\(^{2+}\)-sensitive dyes for measuring Ca\(^{2+}\) dynamics in and between alveolar cells of the lung in the course of the respiratory cycle (1, 15). Other important topics include lipid trafficking, membrane stress failure, and damage and repair (13). Also a broad spectrum of fluorescent proteins and other applications are available that can be used for live cell imaging in mechanobiology, such as fluorescence resonance energy transfer (FRET) or fluorescence recovery after photo bleaching (FRAP), which has been recently reviewed in detail (14).

Unfortunately, it is difficult to use fluorescence live cell imaging when cells are stimulated by globally deforming the elastic growth substrate, which is a widely used method to stretch (or compress) cultivated cells. With this method, only the geometrical center of the growth substrate remains at a constant position relative to the objective of the microscope and usually only this very center portion can be used for imaging. This means that the researcher depends on such an unpredictable circumstance as having the appropriate cell at the right position of the growth substrate. Other positions can of course be used but require recovering the cells after deformation and repositioning them into the optical axis by moving the specimen with the stage of the microscope. For practical reasons this task is often impossible to fulfill and even in successful cases it requires up to several minutes to relocate the area of interest. This impedes the observation of early cellular responses following a mechanical stimulus.

The SC device overcomes this problem with an automated motion compensation mechanism. Within the mechanical limitations described in RESULTS, it works with any desired stretch protocol by setting the appropriate parameters on the graphical user interface of the SC software, enabling cyclic (sinusoidal or other) as well as irregular deformations. Amplitude and rate of stretch can be defined precisely, and pauses can be introduced at any time point (see Figs. 5 and 6 as examples). This allows the user to perform time consuming multichannel image acquisitions, where the stretch has to remain constant while acquiring all channels. The free choice of stretch protocols also includes the possibility of applying compression to the cells. By seeding and cultivating cells on a prestrained membrane, the cells can be compressed in the SC device down to a minimum of \(~60\%\) of the original length by a controlled converging movement of the sliding blocks. The variable width of the membrane chamber in the prestrain holder allows for preparation of a sufficiently prestrained membrane chamber.

The image quality achieved with the SC device can be attenuated by several factors. As shown in Fig. 3, the membrane is compressed perpendicular to the direction of stretch (Poisson effect). The movement of a point caused by this compression depends on its distance from the center of the membrane: it is highest at the center and decreases with increasing distance until it reaches a value of zero at the edges, where the membrane is held by the membrane holders. By selecting objects away from the center, this compression can be reduced. However, even at a position 2 mm away from the center of the membrane perpendicular to the stretch axis and using a \(\times 40\) magnification objective (see Fig. 5) this movement was tolerable, so that motion compensation in this direction was not considered necessary. Another problem is a slight vertical shift of the membrane out of the focal plane during or after stretch, which requires refocusing the specimen. The cause for this displacement is unclear but probably the geometry of the membrane chamber with its side walls (see Fig. 1B) leads to a slight bulging of the membrane. At high stretch rates the membrane supposedly also vibrates immediately after the stretch, although the overlaying bath solution should minimize this effect. The shift in z-direction can either be compensated by manually refocusing (as in all of our experiments) or electronically using an auto focus control in microscopes with a motorized focus and the appropriate software. An example where manual refocusing leads to a gap in image acquisition is discussed below.

Although the signal to the SC motor and the compensation motor is sent simultaneously by the software, some inertia of the mechanical components cannot be excluded. This might result in a delay between stretch and the time that carrier plate is moved to the right position to compensate the lateral displacement. However, if there is such a delay, we were not able to determine it, as it is shorter than the time necessary to refocus.

As neither the deformation of the membrane nor the mechanical parts are strictly following the mathematical algorithm that is used for compensation (see MATERIALS AND METHODS), a certain displacement cannot entirely be avoided. However, even at a generous distance of 3 mm from the center of the membrane, the average lateral displacement is only 0.4% (=12 \(\mu\)m) when stretched to 15% (see Fig. 4C), corresponding to a displacement by \(~1/5\) of the diameter of ATII cells, which were used to test the device.

To demonstrate the practical consequences of the methodological limitations mentioned above and to provide examples for cell biological applications we applied two different stretch protocols to ATII cells. Images were acquired: 1) immediately after a deformation when the cells did not change shape
anymore (as in Fig. 5) and 2) during an ongoing stretch (as in Fig. 6). In the first case we applied one single stretch of 20% to ATII cells within 1 s as an example for a fast stretch in Fig. 5. To further exacerbate the experimental conditions we measured the Ca$^{2+}$ concentration with the ratiometric Ca$^{2+}$ dye Fura-2 (see MATERIALS AND METHODS) and we used a ×40 oil immersion objective. As seen in Fig. 5, A and C, it required 4.6 s from the end of the stretch to measure the intracellular Ca$^{2+}$ increase. The delay was entirely caused by the need to manually refocus the cells. In accordance with the values in Fig. 3, where the membrane compression perpendicular to the stretch axis is shown, a shift of the image perpendicular to the stretch axis can be observed when comparing the position of the measured cells in panels 1 and 2 in Fig. 5. Given that the position of the cells was 2 mm off center position along to the stretch axis and 1 mm perpendicular to it, Fig. 5 shows that even under these very unfortunate experimental conditions an area of 2×4 mm can be analyzed within a time frame of <5 s. The excitation with either 340 nm or 380 nm also demonstrates that the membrane allows excitation in the UV range.

In contrast to acquiring images between changes of cell length, the compensation is naturally less accurate (or requires lower stretch rates) when acquiring a time series during stretch where all mechanical components are in motion. We demonstrate such an image acquisition in Fig. 6, where a strain amplitude of 60% stretch at a strain rate of 1%/s was applied (see Fig. 6A for stretch protocol). The arrows in Fig. 6B (panels 1–4) are pointing at the same cell and demonstrate how much displacement has to be expected despite compensation. As shown in Fig. 6, B and C, this stretch protocol still allowed motion compensation and image acquisition at a cellular resolution in consecutive images. In Fig. 6C we show the analysis of two single cells ~1 mm distant from the center of the membrane, which were recorded at a frame rate of 3 images/s. We used the hydrophilic cytoplasmic dye calcein, which leaks from cells after gross membrane damage to demonstrate single cell analysis for studies on mechanical cell injury. It is obvious that only an approach like this one is able to elucidate individual cell strain amplitudes relevant for damage. The same applies for any other cellular event that depends on the individual gross architecture of a cell.

The major improvement of the SC device described in this study is the automatic compensation of the stretch-induced displacement. We could show that the device is functioning properly with a variety of different stretch parameters and we are convinced that it will prove to be a valuable tool for various microscopic techniques in cell biology and mechanotransduction.

ACKNOWLEDGMENTS

We thank Melanie Timmler and Tatiana Felder for laboratory assistance and Herbert Schmitt for invaluable technical advice.

GRANTS

This work was supported by the Deutsche Forschungsgemeinschaft, Grant D.2428 and the 6th framework of the EU (Pulmonet).

REFERENCES