Shear rheology of a cell monolayer

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Abstract. We report a systematic investigation of the mechanical properties of fibroblast cells using a novel cell monolayer rheology (CMR) technique. The new technique provides quantitative rheological parameters averaged over ∼10^6 cells making the experiments highly reproducible. Using this method, we are able to explore a broad range of cell responses not accessible using other present day techniques. We perform harmonic oscillation experiments and step shear or step stress experiments to reveal different viscoelastic regimes. The evolution of the live cells under externally imposed cyclic loading and unloading is also studied. Remarkably, the initially nonlinear response becomes linear at long timescales as well as at large amplitudes. Within the explored rates, nonlinear behaviour is only revealed by the effect of a nonzero average stress on the response to small, fast deformations. When the cell cytoskeletal crosslinks are made permanent using a fixing agent, the large amplitude linear response disappears and the cells exhibit a stress stiffening response instead. This result shows that the dynamic nature of the cross-links and/or filaments is responsible for the linear stress-strain response seen under large deformations. We rule out the involvement of myosin motors in this using the inhibitor drug blebbistatin. These experiments provide a broad framework for understanding the mechanical responses of the cortical actin cytoskeleton of fibroblasts to different imposed mechanical stimuli.

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1. Introduction

The cytoskeleton is an intricate network of protein filaments [1, 2] which controls the mechanical properties of eukaryotic cells. It is made of three types of filaments: rather flexible intermediate filaments, semiflexible actin filaments and comparably stiff microtubules. In highly motile cells like fibroblasts, actin filaments form a network below the outer cell membrane. This network is largely responsible for vital biomechanical functions, among them the control of cell shape, locomotion and division. The actin cytoskeleton itself is a complex entity. The filaments are highly dynamic and undergo rapid polymerisation or depolymerisation in response to chemical or mechanical stimuli, and in conjunction with polymerisation controlling proteins. The filaments are cross-linked by a large variety of protein molecules, some of which form dynamic cross-links whose properties are controlled by biochemical factors. Some of these cross-linkers, like the motor proteins myosin-II, possess the ability to generate active forces and relative movement between filaments [1, 2]. The actin filament dynamics—polymerisation driven as well as motor driven—are sustained by a steady input of chemical energy derived from the hydrolysis of ATP molecules [1, 2]. All these features make the cell cytoskeleton an active, complex gel. Depending on the mechanical and biochemical conditions, it has the ability to sustain large elastic stresses, can generate internal active stresses and flow, and can undergo rapid remodelling through filament reorganisation. These unique features makes the systematic rheological investigation of the cell cytoskeleton fascinating as well as challenging.
In recent years, important advances have been made in the investigation of the passive rheological properties of live cells occurring at short timescales (see review [3]). Microrheology experiments, which probe at micron scale [4, 5] as well as microplate experiments probing isolated, whole cells [6] have shown broad power-law frequency responses. This behaviour is similar to other gel-like materials [7]. Recently, microplate experiments have shown that cells also exhibit a crossover from linear to nonlinear viscoelastic stiffening response as a function of stress, and independent of strain [8]. Experiments using the optical trap technique have shown that non-adherent cells can also exhibit fluid like behaviour at rates which are slow compared to the binding dynamics of cross-link [9]. Furthermore, recent microplate experiments have given clear evidence of viscoplasticity and kinematic hardening responses in fibroblasts, with simultaneous nonlinear stiffening [10]. These results clearly show the richness in the mechanical behaviour of the cell cytoskeleton. These cell mechanical properties often have vital functional implications [2], [11]–[16].

It is clear from the above examples that a comprehensive investigation of cell mechanics cannot be performed by probing only the linear viscoelastic moduli. It requires the systematic application of specific protocols designed to explore nonlinear responses. Cells being living systems, proper design of the experimental protocol becomes a crucial step as well as a challenge. As our results will show, linearity at small amplitudes cannot be taken for granted at all timescales and needs to be investigated. As the living system can undergo significant mechanical transformations in response to the stimuli used for probing its properties, it is essential to explore the time evolution and steady state behaviour of the responses, and the recovery process. Lastly, in order to identify general characteristic cell responses from ‘random’ cell to cell variations, it is of great advantage to be able to average the data over as many cells as possible.

With these motivations, we have performed a broad and systematic investigation of the rheological properties of fibroblast cells using a novel cell monolayer rheology (CMR) technique. This new technique provides a one-shot averaging of rheological data over $\sim 10^6$ cells, making the experiments highly reproducible and reliable. We show that mechanical behaviour of fibroblast cells can be characterised by a set of very robust, general mechanical responses.

This article is organised as follows. We first describe the technical details and procedures for performing the novel CMR. Following this, we present a systematic set of rheological measurements on normal fibroblasts aimed at characterising the cell responses to different probing techniques. Standard harmonic oscillation experiments (continuous stimulation) are compared with step stress or step strain experiments to investigate the linear and nonlinear regimes that may arise. Cyclic loading experiments are used to explore the evolution of the cell mechanical properties under constant loading cycles starting from a rest state. Further, we perform constant strain rate experiments at different strain amplitudes and strain rates to reveal an unexpected linear stress-strain relation appearing at large deformations. We also explore the effect of an underlying preload on the response to small amplitude harmonic oscillations. Finally, the results obtained from normal cells are compared to those from cells whose cytoskeleton has been biochemically modified. These experiments demonstrate the contributions arising from dynamic elements in the cytoskeleton and shed light on to microscopic mechanisms. The systematic nature of our investigation provides a broad, quantitative, understanding of the main mechanical responses of the fibroblast cells under mechanical stimulation.
Figure 1. A schematic representation of the rheometer set-up designed to perform CMR. A monolayer of single cells is held between two glass discs in a plate-plate geometry. A commercial rheometer performs shear rheological measurements on the monolayer. The fluid outlet is used to change the cell culture medium in order to treat the cells with biochemical agents which modify the cytoskeleton.

2. CMR

The rheological measurements are performed using a Modular Compact Rheometer (MCR-500) from Anton–Paar GmbH, which we modified to enable CMR as shown in figure 1 (European patent application [17]). The measurements are performed in a coaxial plate-plate geometry, where the cells are held between two parallel glass discs. The top plate (50 mm diameter) is attached to the measurement head of the rheometer and the bottom plate (70 mm diameter) to the base via metal mounts. An outlet drilled into the bottom plate is used to exchange the medium between the plates without changing the gap or mechanically disturbing the cells. A microscope which can scan along a radial direction allows optical observation of the cells during measurements and also the estimation of cell density. Images can be recorded on to the computer using a CCD camera at a maximum rate of 15 frames s$^{-1}$. The gap between the plates can be adjusted to within $\pm 1 \mu$m. The temperature of the sample is maintained using a Peltier unit at $25 \pm 0.1 ^\circ\text{C}$. A picture showing the modifications to the set-up is shown in figure 2.

Performing rheological investigations on a monolayer of isolated cells involved several technical and procedural challenges. First of all, the gap between the plates has to be about the same as the size of a single isolated cell, which is only about $10 \mu$m. Moreover, since cells are very soft objects, the total measurement area of the plates must be of the order of $20 \text{cm}^2$ for the rheometer to be able to resolve the torques. One of the major problems then is in achieving the required parallellity between the two plates of the rheometer. The opposing faces must be parallel
Figure 2. Photograph of the CMR set-up. The levelling screws (±1 \(\mu\)m precision) are used for fine corrections to the alignment, if the plates are not parallel after following the procedure described in the text [17].

to each other within 1 \(\mu\)m/10 cm = 10\(^{-5}\) rad. This problem is solved by using special polished glass plates with a surface flatness of \(\sim 500\) nm, plus an appropriate preparation procedure. This and other procedural details are discussed in the following subsections.

2.1. Mounting and optically adjusting the glass plates

The steps involved in mounting the plates and ensuring the parallelity between them are as follows.

1. After thorough cleaning, the top glass plate is carefully placed on top of the bottom glass plate taking care that no dust particles are trapped in between the two plates. The parallelity between the plates can be easily verified by observing the interference fringes formed by a distant, broad, white light source (fluorescent lamp). The interference between the reflections from the two inner surfaces of the plates results in periodic coloured fringes (fringes of equal thickness) [18]. The fringes represent contours of equal gap between the two plates and the spacing between adjacent lines is proportional to the spatial gradients in the gap thickness. Using this method we ensure that the plates, when placed in contact, are parallel to within 1 \(\mu\)m.

2. Once the glass plates are satisfactorily in contact with each other, they are placed on the rheometer without separating them. The bottom glass plate is fixed to the bottom metal mount of the rheometer (see figure 3(a)).

3. The top metal plate of the rheometer is brought down until it makes contact with the top glass plate. This is automatically done by the rheometer as a normal zero-point setting. The metal plate is then locked in position to prevent rotation (figures 3(a) and (b)).

4. The top metal plate is then glued to the top glass plate using the optics grade, ultraviolet light curable adhesive Vitralit 6129 (Panacol–Elosol GmbH) (figure 3(b)). This is a
Figure 3. Schematic showing the various steps involved in preparing the cell monolayer. (a) The two optically flat glass plates are placed in perfect contact, (b) a UV curable adhesive is used to glue the top glass plate to the rheometer head, (c) solutions of adhesion promoting proteins used for surface treatment or the cell suspension can be loaded between the plates using a pipette, (d) the cells, after loading, are allowed to sediment, and (e) the top glass plate is lowered until the cells are slightly compressed.

thick adhesive with a very low thermal expansion coefficient of $36 \text{ ppm K}^{-1}$, which later can be easily removed by leaving overnight in acetone. The glue is cured by exposure for a few minutes to UV light with a wavelength of 365 nm and an intensity of $\sim 100 \text{ mW cm}^{-2}$. This procedure ensures excellent parallelity between the two glass plates as the system is assembled with the two glass plates in perfect contact. A picture of the final set-up is shown in figure 2. The levelling screws shown here are used for any final corrections to parallelity that may be required. The laser can be used to obtain an interference pattern, as an alternate method for aligning the plates.

After fixation, the parallelity is usually within 2–3 $\mu$m over the entire plate. Once fixed, the angular position of the top plate is locked and only small amplitude oscillations are applied about this position during measurements. The fixed top plate can be lifted up to 1 cm and brought back without any significant loss of parallelity.

2.2. Coating the plates with adhesion promoting proteins

Once the glass plates are positioned, they are coated with the adhesion promoting protein fibronectin (Sigma-Aldrich). For this a final solution of the protein at a concentration of $30 \mu g \text{ ml}^{-1}$ in phosphate buffer solution (PBS) is prepared. Introducing the fibronectin solution
is straightforward. Since the plates are clean and dry, at a spacing of $\sim 200 \mu m$, capillary forces readily suck the solution into the gap (figure 3(c)). The fibronectin solution is left between the plates for 1 h. Then the top plate is brought down to a nominal gap of 10 $\mu m$, which pushes the excess solution out. To remove it completely from the plates, the fibronectin solution is sucked with a pipette. Once the protein is adsorbed, the plates are rinsed three times using PBS by raising and lowering the plates as before.

2.3. Preparation of cells

All experiments are performed on Swiss 3T3 fibroblasts [19, 20] from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) [21]. After defreezing cells stored in liquid nitrogen, fibroblasts are grown for at least a week and no longer than 2 months, following standard protocols. The medium used for regular culture is Dulbecco’s modified Eagle medium (DMEM), with glucose 4.5 g l$^{-1}$ and 10% fetal bovine serum (FBS). Experiments are performed in Iscove Medium with HEPES 25 mM and lyso-phosphatidic acid (LPA) (Sigma-Aldrich) at a concentration of 0.5 $\mu M$ (instead of serum, which contains LPA [22]). Prior to an experiment, cells are detached from the flask by 5 min exposure to a trypsin solution, then centrifuged at 100 g for 2 min in regular culture medium, and finally resuspended at the desired concentration in the medium used for the experiment. All cell culture reagents are from Gibco (Invitrogen, Carlsbad, CA, USA).

2.4. Loading the cells between the plates

The cell suspension is introduced in a similar fashion as the fibronectin solution discussed earlier. Before lowering the top plate, it is mandatory to wait for about 10 min. The reason is that the cells must be allowed to sink down and stick to the bottom plate, or else the outward movement of the liquid induced by bringing the plate down removes some of the cells. This must be avoided, as we need a very high cell density. Waiting for too long before bringing the plate down is also undesirable, as the cells will spread excessively on to the bottom plate and are then unable to attach sufficiently to the top plate. This is known from the single fibroblast experiments described in [8]. After a prudent time, the top plate is brought down until most of the cells are slightly compressed (figures 3(d) and (e)). An image of the cells adhering to the plates, and under slight compression, is shown in figure 4. The compression of the cells is easily observed by measuring the increase in their diameters from the recorded images as is shown in figure 5. The cells are left in this state for 1 h to allow them to adhere to both plates, before measurements are performed. After this period, the fraction of the cells that are firmly adherent to both the plates can be roughly estimated as follows: (i) by applying a step shear strain of about 20% and observing the cell deformation and (ii) by observing the cell geometry at the upper and lower boundaries, where adherent cells have roughly constant diameter. This fraction is typically of the order of 50% of the cells for a gap of about 8 $\mu m$ and varies a little from cell preparation to preparation and with plate gap. The responses reported here are unaffected by the exact fraction of adhering cells as long as the stresses are above the resolution of the rheometer (also see gap effect in appendix). Since the main focus of the present work is on reproducible, general aspects of nonlinear behaviour, the precise value of the moduli are not relevant. We have therefore refrained from correcting stress values for the fraction of adhering cells.
Figure 4. Image of a typical cell monolayer which is gently pressed between the two glass plates. The bar represents 50 µm.

Figure 5. A sequence of images showing the cells (a) after they have sedimented and (b) after they are gently pressed between the glass discs by lowering the top plate. Note that the diameter of the big cell increases as it is gently squashed. The gap between the plates is such that the smaller cells remain unperturbed.

2.5. Method for introducing drugs

Drugs which alter the cytoskeletal structure can be easily introduced by adding them at a final concentration along the rim of the plates and sucking the medium between the plates through the outlet in the bottom plate (see figure 1). Usually, the gravitational flow is enough to ensure smooth exchange of media within a few minutes without causing any significant flow disturbances to the cells. The cells are observed throughout this process to ensure that the flow does not change the cell density or alter their morphology.

2.6. Top plate with annular ring

If large shear deformations are to be imposed in order to explore the nonlinear regime, a difficulty inherent to the geometry arises. In a plate–plate configuration the deformation field is not uniform: the shear deformation increases proportional to the radius. For most experiments this effect can be neglected, since the dominant contribution to the torque comes from the strongly sheared cells on the outer edge. If better resolution is desired, a simple solution is
available. On the top plate an inner circle with a depth of $\sim 100 \mu m$ is carved away with a standard milling machine. Since the cells are at most $\sim 40 \mu m$ large, only those located on the outer, non-processed section will be in contact with both plates and contribute to the measurement. We have performed measurements with such a glass plate to confirm the feasibility of the approach. Qualitatively the responses are the same.

2.7. Major advantages of the CMR technique

The novel CMR technique provides us with the following major advantages. (i) Probing a large number of cells ($\sim 10^6$) provides a one shot average of the mechanical properties, which can vary strongly from cell to cell in a given population. This makes the experiment highly reproducible from one cell preparation to another. (ii) The nature of cell-substrate adhesion can strongly influence the mechanical properties. Our technique allows the control of cell-substrate adhesion by using functionalised glass plates while at the same time keeping the cell geometry relatively simple (see figure 4). (iii) A large variety of probing techniques—oscillatory probing at varying amplitude or frequency, controlled stress or strain experiments, ramp experiments, and large amplitude deformations are all possible, a necessity for investigating complex materials.

3. Experimental results

3.1. Harmonic oscillation experiments

We begin our investigation by characterising the response of the cell monolayer to imposed sinusoidal strain oscillations. Our intention is to explore the extent of linearity of the response from this living system. We then study the frequency response of the system at small amplitudes.

3.1.1. Amplitude sweep. For strain amplitudes between 1 and 10% and a frequency of 1 Hz, increasing the strain amplitude results in a less and less elastic behaviour as shown in figure 6. The storage modulus goes roughly as $G' \sim \log (1 / \Delta \gamma)$, whereas the loss modulus $G''$ becomes plateau-like for $\Delta \gamma < 3\%$. Thus, a linear regime in a strict sense does not exist in this measurement range.

3.1.2. Frequency sweep. Figure 7 shows a frequency sweep data obtained at an amplitude of 2%. Both $G'$ and $G''$ show a power-law behaviour throughout the studied range. The loss tangent remains approximately constant as shown. The power-law behaviour observed here is in excellent agreement with that previously reported using microrheology and single cell techniques [4, 6]. The curves for $G'$ and $G''$ remain almost parallel throughout the studied frequency range.

3.2. Stress relaxation, recovery and creep experiments

As cells are complex living materials which can actively respond to different types of mechanical stimuli, we now proceed to investigate their response to stepwise loading by performing relaxation and creep experiments. The two types of step loading experiments are then compared to study the linear and nonlinear cell response that may arise.
**Figure 6.** Amplitude sweep at a frequency $\nu = 5$ Hz. The storage modulus $G'$ (left) and the loss modulus $G''$ (right) are shown. Whereas $G''$ shows a clear plateau below 3%, the storage modulus increases steadily as the amplitude is reduced down to 0.3%.

**Figure 7.** Shear moduli $G'$, $G''$ (top plot) and loss tangent $G''/G'$ (lower plot) as a function of frequency for a fixed amplitude $\Delta \gamma = 2\%$.

For comparing the relaxation and creep compliance of the cells we have devised the protocol shown in figure 8. First, we impose a 5% step strain and measure stress relaxation during 10 min (S1 in figure 8). Then, a large step strain from $\gamma = 5\%$ to $\gamma = 50\%$ is applied and a second relaxation curve is measured (S2). After 10 min at 50% strain, the monolayer is unloaded in a stepwise fashion and the stress kept at zero for 35 min in order to measure strain recovery (R1). Next, we perform two subsequent creep experiments, the first one at a low stress
Figure 8. Stress relaxation, strain recovery, and creep measurements performed sequentially. The input step functions are shown as grey shaded areas. Small and large strain or stress steps are applied to explore and compare the linear and nonlinear responses.

of 5 Pa (C1) and the second one at a high stress of 25 Pa (C2). The stress values are chosen from the previous relaxation experiments so that S1 can be compared with C1, and S2 with C2. Finally, a second full unloading to $\sigma = 0$ is performed (R2). The whole procedure takes about 90 min, which is a reasonable time for a measurement at 25 $^\circ$C. S1 and C1 are expected to be close to a linear regime, while the large steps S2 and C2 should reveal effects of strong nonlinearities. The different responses are discussed and compared below.

3.2.1. Stress-relaxation. Figure 9 shows stress relaxation curves obtained at constant strain, after a 0% $\rightarrow$ 5% and a 5% $\rightarrow$ 50% strain step. As expected from the frequency sweeps discussed earlier, the relaxation cannot be described by a single exponential and is close to a power-law. Remarkably, no significant nonlinearity is observed as a function of the applied strain amplitude. Normalising by the initial values suffices to collapse the curves.

3.2.2. Strain recovery at zero stress. After a deformation and subsequent unloading to zero stress, do irreversible strains remain? To decide on this, we perform the following experiment. After imposing a 50% shear for 10 min, the stress $\sigma$ is taken to zero and the time evolution of the strain $\gamma$ is recorded (R1 in figure 8). Figure 10 shows the extent of recovery $\gamma(-0) - \gamma(t)$, where the strain prior to the unloading is subtracted. In the first recovery experiment, R1, the strain recovers from 50% to 20% in a 2000 s time period and is still slowly recovering. At this pace, a full recovery would require many hours. The question seems deemed to remain
Figure 9. Stress relaxation at constant strain for the two different strain steps shown in figure 8. The stress $\sigma$ is divided by its value right after the step, $\sigma_0$, and shown as a function of the time elapsed after the strain step. Other than the prefactor, no significant differences can be observed between the two curves. The apparently larger scatter in the curve at $\gamma = 5\%$ is due to the normalisation by $\sigma_0$. The scatter in the stress $\sigma$ is essentially constant throughout the experiment.

Figure 10. Strain recovery at zero stress obtained from the responses R1 and R2 in figure 8. The extent of recovery $\gamma(-0) - \gamma(t)$ as a function of time are shown, where $\gamma(-0)$ is the strain prior to the unloading.

unanswered, and not only for technical reasons; the time for complete strain recovery is close to typical cell lifetimes of $\sim 10^4$ s.

3.2.3. Creep experiments. Figure 11 shows the compliances from the creep experiments C1 and C2. The compliance functions are defined as $J(t) = (\gamma(t) - \gamma(-0))/(\sigma - \sigma(-0))$, where $\gamma(-0)$ is the deformation prior to the stress step, $\sigma$ the imposed constant stress during creep, and $\sigma(-0)$ the stress prior to the step. Experiment C2, performed at a large stress of $\sigma = 25$ Pa, gives a significantly larger compliance for times shorter than 100 s. Remarkably, at longer times it approaches the small-stress compliance.
Figure 11. Creep compliances $J(t)$ for experiments C1 (5 Pa) and C2 (25 Pa) in figure 8. The behaviour obtained at large stress and short timescales is significantly different from that at small stresses. Unlike in the relaxation and recovery experiments mentioned earlier, the curves cannot be collapsed by a simple scaling anymore.

3.2.4. Convolution of the relaxation modulus and compliance. We now compare the responses obtained from the relaxation and creep experiments, to assess the linearity of the response. Linear behaviour is given by Boltzmann’s superposition principle: the stress is a linear function of the strain history,

$$\sigma(t) = \int_0^{\infty} d\tau \, G(\tau) \dot{\gamma}(t-\tau),$$

where the function $G(t)$ is the relaxation modulus [23]. It is straightforward to show that

$$t = \int_0^{t} d\tau \, G(\tau) \, J(t-\tau), \tag{1}$$

holds [23]. With this convolution relation one may decide whether the material behaves as a passive, linear system.

We proceed as follows. The relaxation moduli are obtained from the relaxation experiments S1 and S2 as $G(t) = \sigma(t)/(\gamma - \gamma(-0))$, where $\gamma$ is the imposed constant strain and $\gamma(-0)$ is the strain prior to the strain step. We numerically convolute the measured relaxation moduli with the creep compliances to assess the validity of equation (1). The advantage of the procedure is that we work directly with the measured response functions, instead of ad hoc choosing a fitting function. We convolute $G(t)$ from S1 with $J(t)$ from C1 which corresponds to the small stress regime, and $G(t)$ from S2 with $J(t)$ from C2 for the large stress regime as shown in figure 12. Both convolutions deviate significantly from the linear behaviour at short times. For intermediate times of $\sim 10$ s the small-stress convolution approaches identity. The large-stress convolution shows nonlinear behaviour at these intermediate times, but becomes a linear

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Figure 12. Comparison between the stress relaxation and creep responses from the experiment shown in figure 8 is performed by the convolution $\int_0^t \, d\tau \, G(\tau) \, J(t-\tau)$ of the conjugate response functions. Within linear response theory, the integral is identically equal to $t$. Grey symbols: convolution of $G(t; 5\%)$ with $J(t; 5 \text{ Pa})$. Black symbols: convolution of $G(t; 50\%)$ with $J(t; 25 \text{ Pa})$. Black dashed line: identity. Significant deviations from linear behaviour are seen only at short times. For times beyond 100 s the response approaches linearity, even for large step deformations of 50%.

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loops is a very robust feature of the fibroblast monolayer and the same behaviour is observed at the single cell level [24].

The effects of a cyclic straining are reversible. When the cells are maintained at zero strain for more than 10 min and the experiment repeated, the new virgin curve and the limit cycle are quantitatively similar to the previous ones (data not shown).

### 3.3.2. Amplitude dependence

Figure 14 shows single loading/unloading cycles with different amplitudes. A rest time of 10 min elapses between each cycle, which ensures a sufficient stress relaxation. The stress-strain curves share a common envelope. This indicates that the rest time sufficed to recover the virgin state. The slope $d\sigma/d\gamma$ reaches a roughly constant value after the initial $\sim 10\%$ strain. For small amplitudes the unloading response is very similar to the loading response, essentially a point inversion of the latter. As the amplitude is increased, the unloading response changes its shape noticeably. When unloading, again a linear stress-strain relation is obtained as the strain is lowered from its peak value.

### 3.3.3. Rate dependence

We perform single cycles at different rates, waiting 10 min between each cycle. Changing the rate changes the overall slope of the curves, as can be seen from figure 15. The hysteresis loops are also seen to become wider.
3.4. Effect of the average stress on the viscoelastic moduli

In earlier sections, we have performed a detailed characterisation of the cell response to oscillatory and cyclic ramp experiments by varying either the deformation rate or the amplitude. These experiments were performed on cells which were at an initial state where the stress and strain are almost completely relaxed. It is interesting, however, to investigate how the cell rheological properties are modified when the cells are under a nonzero stress or strain condition.

For this, we perform the following experiment. Starting from a zero stress, zero strain condition, we apply a fast strain step to the cells. The average strain is then maintained constant while a small amplitude strain oscillation (2% and 5 Hz) is superimposed; \( \gamma(t) = \langle \gamma \rangle + \Delta \gamma \cos(\omega t) \). After the strain step, the average cell stress will relax with time as \( \sigma(t) = \langle \sigma \rangle(t) + \Delta \sigma(t) \sin(\omega t) \). Measurements are performed when the stress relaxation is slow compared to the frequency of the imposed oscillations. In this way, by applying different strain steps, the moduli can be measured for a large range of stress or strain values. The procedure, though unusual for passive materials,
Figure 15. Rate dependence obtained by applying single constant strain rate cycles with a 10 min rest time between cycles. Top: strain $\gamma$ as a function of time. Bottom: stress-strain loops for the cycles shown above.

has proved successful to study stiffening responses in biomechanics [25]. The results are shown in figure 16. It can be seen that both moduli, $G'$ and $G''$, stiffen as a function of the average stress above a threshold stress. i.e. $G' = G'(\langle \sigma \rangle)$ and $G'' = G''(\langle \sigma \rangle)$ above the threshold. In particular, stiffening can be observed during stress relaxation at a constant strain for $\langle \gamma \rangle = 100$ and 120%.

Above the threshold, both moduli follow a power-law with an exponent of about 0.7.

3.5. Drug experiments

After characterising the normal fibroblasts, we now discuss a series of experiments aimed at investigating the role of different cytoskeletal elements like actin filaments and myosin motor proteins in the mechanical responses detailed above. Moreover, we perform, for the first time, experiments demonstrating major qualitative differences between the mechanical responses of an active, living cell to a cell which is made passive, permanently crosslinked.

3.5.1. Actin depolymerisation. In order to investigate the contribution of the actin network in the cells to the mechanical properties mentioned above, we treated the cells with an actin filament depolymerising drug latrunculin-A [26]. For this, we first characterised the normal cells by performing step-strain experiments and then introduced the drug at a final concentration of 0.2 $\mu$g ml$^{-1}$ without mechanically perturbing the cells using the method discussed in section 2.5.
**Figure 16.** Top: a schematic of the imposed deformation history. Fast small amplitude oscillations at 5 Hz and 2% amplitude are superimposed over constant average strain values $\langle \gamma \rangle$. Bottom: the shear moduli $G'$ and $G''$ measured as a function of the average stress $\langle \sigma \rangle$ and strain $\langle \gamma \rangle$. A range of stress values are obtained during the stress relaxation which follows each strain step and are shown using different symbols. The dashed line corresponds to a $\sim x^{0.7}$ dependence. Inset: typical relaxation of the average stress $\langle \sigma \rangle$ after a large strain step, normalised by the value after the step. Measurements are performed when the stress relaxation becomes slow compared to the frequency of the imposed oscillations.

We observe that a 10 min exposure to the drug at 25°C induces a marked drop in the stiffness of the monolayer, as shown in figure 17. For a given shear, the stresses are two orders of magnitude smaller, barely resolvable by the rheometer.

3.5.2. Comparison of living and ‘fixed’ cells. Due to the active nature of the cytoskeleton, it is interesting to try and elucidate the contributions from the dynamical factors to the mechanical responses mentioned above for normal cells. With this in mind, we attempted generating purely passive cells (or dead, equilibrium system) by exposing the cells to a fixation agent glutaraldehyde. This process binds the network in such a way that all dynamical processes like...
Figure 17. Top: the effect of actin depolymerisation is studied by applying a large step strain (25–50%) and measuring the stress relaxation. Bottom: stress relaxation curves obtained from the same monolayer for normal cells and for cells treated with latrunculin-A (0.2 µg/ml) to disrupt the actin network.

Filament polymerisation-depolymerisation, kinetics of motors and crosslinking proteins, etc are arrested, though preserving the cytoskeletal structure [27].

As can be seen from figure 18, the typical mechanical behaviour of normal cells is dramatically altered after fixation of the cells by a 10 min. exposure to a 0.1% glutaraldehyde solution. Most notably, the large-amplitude linear behaviour of the limit cycle is completely abolished by the treatment. The response of the passive, fixed cells shows a positive curvature \( \frac{d^2\sigma}{d\gamma^2} \). Due to this dramatic stiffening the stress at 100% shear increases by an order of magnitude. To further assess the effect of glutaraldehyde on cytoskeletal structure, we compare the numerical derivative \( \frac{d\sigma}{d\gamma} \) of the stress-strain relation obtained after fixation with previous results on single cells (figure 18, inset). As a function of stress, the slope \( \frac{d\sigma}{d\gamma} \) is remarkably similar to the stiffening master-relation described in [8]. This agreement between ‘dead’ (fixed) and living samples conclusively proves that the stiffening response in living cells reported in [8] is due to the nonlinear elasticity of the cytoskeletal network, independent of biological processes such as e.g. signalling, restructuring, crosslink-dynamics or motor activity. Moreover, since glutaraldehyde fixation does not significantly alter the elastic response, its ‘stiffening’ effect must actually be to slow down inelastic flow of the cytoskeleton—presumably by preventing detachment of crosslinks. Glutaraldehyde fixation has a similar effect in single fibroblasts under uniaxial elongation [10].

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3.5.3. Inhibition of myosin-II motors. The glutaraldehyde experiment described in the previous section shows that the cell response is drastically altered when the cells are made passive or dead with fixed crosslinks and filaments. However, it is not clear as to what extent the dynamics of motor molecules are involved in controlling cell mechanical properties. A separate experiment is required to explore this aspect.

In order to assess the role of myosin-II motors on cell mechanics, we inhibit them using the specific drug blebbistatin [28, 29]. Ramp experiments, like the one discussed in the previous section, do not reveal any qualitative differences compared to normal cells (data not
We still observe a linear stress-strain relation after the initial loading or unloading, although the moduli $G'$ and $G''$ are slightly lower compared to normal cells. Previously reported microrheology experiments have shown a qualitative difference in the frequency response obtained from blebbistatin treated cells [5]. In order to compare our results with this report we performed frequency sweeps using the present technique and obtained the results shown in figure 19.

As a control, we perform an amplitude sweep and frequency sweep on the untreated cell monolayer. We then add the drug at a final concentration of 150 micromolar to the cells. After a waiting time of 10 min the amplitude sweep and frequency sweep is repeated. No significant difference is observed in any of the two experiments, aside from a prefactor. To rule out the possibility of the drug not working, we tested its effect on arresting the oscillatory dynamics of freely suspended fibroblasts [30].

Figure 19. Frequency sweeps at an amplitude $\Delta \gamma$ of 2%. The moduli and loss tangent obtained for normal cells (open symbols) and for cells exposed to 150 $\mu$M blebbistatin (solid symbols) are shown.
4. Discussion

4.1. Summary of experimental results

The CMR technique is a very versatile method for probing the complex rheological properties of cells. Linear viscoelastic properties, nonlinear responses which arise under different loading conditions, temporal variations, and inelastic flow properties are all accessible using this method. In the past, rheological investigations on collections of living cells often addressed cells inside a protein matrix, such as collagen gels [31, 32]. Interpretation of the data obtained in this way is difficult, as the extracellular matrix itself has mechanical properties very similar to those of cells. In our case, the external medium is a Newtonian liquid with a negligible viscosity. Another approach are sedimented cell pellets [12]. Our cell monolayer technique has the advantage of a clean geometry where cells are mechanically independent from each other. Therefore, each measurement gives an arithmetic mean over \( \sim 10^6 \) cells, making the experimental results highly reproducible and easy to perform compared to single cell techniques. Functionalising the plates using adhesion promoting proteins allows the cells to form specific cell-substrate adhesion, at the same time maintaining a simple overall cell geometry when compared to spread cells used in microrheology studies. As we demonstrate, the role of different cytoskeletal components and the comparison between active and passivated cells can also be performed using biochemical techniques, without mechanically perturbing the cell monolayer. Incidentally, the results obtained do depend on the gap between the plates. An optimum gap is chosen for the experiments so that the cells are not too strongly compressed (see appendix).

Harmonic oscillation experiments clearly show that a strict linear regime does not exist for the storage modulus \( G' \) even at the smallest strain amplitude of about 0.2%, at a frequency of 5 Hz (figure 6). An amplitude range of 0.2–2% may be considered approximately linear. The frequency sweep in this approximately linear regime (figure 7) exhibits a clear power-law increase of both \( G' \) and \( G'' \) over three decades of frequency (10\(^{-2}\)–30 Hz). Moreover, the \( G' \) and \( G'' \) curves remain parallel throughout the frequency range. There is no crossover from an elastic to viscous behaviour.

Relaxation, creep and recovery experiments performed by applying step strains and step stresses, respectively, reveal the existence of a continuum of relaxation times in the system (figure 9). The stress relaxation continues even at the longest observation times (10 min). The relaxation spectra obtained at different loading strains are different only by a constant scaling factor (figure 9). Creep as well as strain recovery experiments (figures 8 and 11) too show long time recovery effects (>20 min). Convolution of relaxation moduli and creep compliances shows that initially nonlinear responses become linear after a certain time, which amounts to 100 s for experiments performed at \( \sim 100\% \) strain (figure 12).

Cyclic loading experiments show an evolution of the initial response towards a steady state ‘limit cycle’ (figure 13). When a rest time of 10 min is allowed the system recovers the initial ‘virgin response’. This can be observed for different loading rates.

Varying the strain amplitude reveals a surprising feature of the cell response. The response which is nonlinear at small strains becomes almost perfectly linear as the strain increases (figure 14). This entry to linearity at large amplitudes is observed for the studied strain rates of \( 10^{-3} \)– 1 s\(^{-1} \) (figure 15). On reversing the sense of strain rate (unloading) the cells again exhibit an initial nonlinear response and a later linear response. Within the explored range, with increasing strain rate the linear modulus and the hysteresis increase.

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The viscoelastic moduli as a function of stress show a power-law stiffening response above a threshold stress (figure 16). Both moduli stiffen as a function of stress with a very similar power-law exponent. The threshold stress values are also similar for both moduli.

Drug experiments reveal the following. When actin filaments are depolymerised, the cells are transformed from viscoelastic objects to almost purely viscous ones. When the cell cytoskeleton is permanently crosslinked using a fixation agent, the large amplitude linear response is replaced by a strong stiffening response. Inhibiting myosin motor molecules, on the other hand, does not produce any qualitative change in the rheological behaviour of the cells.

4.2. Comparison of different results

In this section, we compare the different results discussed above in order to reveal some general trends in fibroblast cell mechanics and compare them with the recent literature.

Cell response timescales: the frequency scan performed using small amplitude harmonic oscillations (figure 7) is in excellent agreement with the relaxation spectra obtained from the step strain experiments (figure 9). Both experiments show that there exists a continuum of relaxation times in the system. The relaxation continues to happen even at the longest observation times. Power-law relaxation spectra have been observed for a variety of cell types using microrheology and atomic force microscopy techniques [3, 4, 33, 34]. This, apart from validating the CMR technique, shows that the collective response of $10^6$ cells is indeed comparable to single cell responses obtained under different conditions. Recovery spectra recorded at zero stress gives a similar picture for the timescales involved (figure 10), with the strain recovery continuing even at long times (>20 min).

Constant strain rate experiments performed at different loading rates show an increase in the slope of the stress-strain relation with increasing rate (figure 15). This is expected for viscoelastic materials, and can in principle be understood in terms of linear viscoelasticity.

Linear and nonlinear regimes: the stress relaxation curves recorded at different strains and recovery recorded at different initial strain values collapse to the same curve on normalisation using the respective initial values. Thus, these responses are independent of the initial loading condition, as expected for a linear regime. In the case of creep experiments, at large stresses a qualitatively different response is observed (see figure 11). However, as the convolution of compliance and relaxation modulus conclusively shows, the cell monolayer asymptotically becomes a linear, passive system for times longer than $\sim 100$ s. This is even more remarkable as the strains involved are of the order of 100%.

Another counterintuitive behaviour is observed in cyclic constant-rate loading experiments (figures 14 and 15). The response which is nonlinear at small strains becomes almost perfectly linear at large strains. On reversing the sense of deformation, the response is nonlinear at large strains and becomes almost linear at lower strain values. These effects are more clearly seen at lower strain rates (figure 15). Thus, the initial response of the cells to large constant rate straining is nonlinear and there is a crossover to linear behaviour as the straining is continued. This observation is very similar to that observed in single cells using the microplate stretching technique [10, 24].

Stress stiffening: harmonic oscillation experiments performed on cells under nonzero average stress show a power-law stiffening response for both $G'$ and $G''$ as a function of the average stress (figure 16). This strain independent stress-stiffening observed in our monolayer
shearing experiment is remarkably similar to that previously observed in single cell stretching experiments [8]. Similar stiffening responses have also been reported for tissues [24]. Unlike the increase in the modulus as a function of rate observed in figure 15, this stiffening is a nonlinear effect. When the cell cytoskeleton is made permanent using a fixing agent, the cell exhibits a stiffening response over a very wide amplitude range replacing the linear stress-strain relation observed in normal cells (figure 18 (inset)).

Correlation between $G'$ and $G''$: it is also interesting to compare the correlation in the behaviour of the two moduli $G'$ and $G''$ in the different experiments. In frequency scans, both moduli increase with frequency with very similar power-law exponents for the entire range of frequencies (figure 7). The loss tangent remains almost constant as previously reported using other techniques [4, 34]. Such a strongly correlated behaviour is even more striking in the stress-stiffening response (figure 16). Here, the crossover threshold as well as the exponent for the power-law stiffening response are very much comparable for both moduli. However, in experiments probing the frequency response of single cells in suspension using an optical stretcher technique, a crossover from elastic to viscous behaviour is observed as the frequency is reduced [35]. Presumably this reflects the differences in boundary conditions, which should lead to different cytoskeletal structures.

Comparison of normal cells and biochemically modified cells: depolymerisation of actin filaments causes the cells to lose their elastic properties almost completely (figure 17). This transformation shows that the actin network plays an important role in defining the mechanical properties of these cells. Large amplitude, constant rate loading experiments performed on normal living cells and cells with fixed cytoskeleton (permanent crosslink and filament structure) produce completely different cell responses (figure 18). The linear regime which is clearly observed in normal cells for a wide range of loading rates is replaced by a stiffening response in fixed cells. This experiment conclusively proves that dynamic crosslinks, or filaments are necessary for the large amplitude responses exhibited by normal cells. Motor proteins, which also form a class of dynamic crosslinks with the ability to generate active forces and relative motion between filaments, do not appear to play a prominent role in the studied responses as discussed in the text. A comparison of the frequency sweeps on normal cells and that performed on cells with inhibited myosin motors shows only a slight reduction in both $G'$ as well as $G''$ upon drug treatment, while retaining the qualitative features of the normal cell response (figure 19). This result, which we obtain by shearing a cell monolayer, is qualitatively different from microrheology experiments performed using optical tweezers on single cells [5]. In the latter case, the loss modulus $G''$ becomes independent of frequency when myosin motors are inhibited.

4.3. Conclusions and speculations

Taken together, our results lead to the following picture of cell mechanics. The actin cytoskeleton defines cell mechanical behaviour via an interplay between nonlinear elastic behaviour and linear inelastic behaviour. At short timescales, crosslinks stick and the network responds elastically. Due to its nonlinear elastic properties, it stiffens at a large average stress. This stiffening response is by now well established as a general feature of biopolymer networks [3, 25, 36], and can even be observed in vitro in crosslinked actin networks [37]. Proposed explanations range from entropic stretching [37, 38] to enthalpic bending [8, 39, 40]. If the stress is further increased, the extent of linear inelastic flow increases dramatically in a
non-Newtonian fashion. The microscopic mechanism for this inelastic response is most likely crosslink slippage, but may also involve filament growth. The inelastic flow regime goes hand in hand with a remarkably linear stress-strain relation. Such a behaviour is by no means unique to biological cells: under the name of kinematic hardening [41, 42], it is commonly observed in composite alloys [43, 44], as well as in rubbery polymers [45] and granular materials [46]. Microscopic understanding of this hardening response may well be a crucial step for the further development of our knowledge of cell mechanics.

Living cells may respond to the sudden mechanical stimulus by undergoing a transient reorganisation of its internal structure. Subsequently, since the input is kept constant after the initial stimulus, the system could evolve towards a ‘steady state organization’ with time. This may explain why a strict linear regime is elusive in the amplitude sweeps (even at about 0.1% strain), where the cell is under continuous mechanical perturbation.

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Appendix A. Effect of gap thickness

Changing the distance between plates has an effect on the monolayer response. We study this by means of oscillatory and stress relaxation experiments. Because of this gap-dependence, the experimental protocol should always begin with a gap-optimisation.

A.1. Frequency sweep at 1% amplitude

Figure A.1 shows a set of frequency sweeps performed at a strain amplitude $\Delta \gamma = 1\%$, starting from an the initial gap of 6 $\mu$m. A weak dependence of $G'$ with frequency can be seen. When measurements are performed as soon as the monolayer is loaded between the plates and again after one hour, both $G''$ as well as $G'$ increase by about a factor of two. This may be attributed to cytoskeletal restructuring which is expected to occur as the cells develop focal adhesion complexes with the fibronectin coated plates. Similar effects can be observed on single-cell experiments [33]. Increasing the gap from the initial 6 to 22 $\mu$m lowers both moduli by over a decade, though their frequency dependence is not significantly affected. This effect is most likely due to a decrease in the number of cells contacting both plates.

A.2. Step strain and stress relaxation

Stress relaxation experiments performed at different gaps agree with the results shown above. The monolayer is much stiffer at smaller gaps (see figure A.2). As discussed earlier, this effect is most likely due to a larger number of cells contributing to the measurement. At very small gaps (about 4 $\mu$m), the data becomes noisy and the relaxation curve behaves in an unconventional
Figure A.1. Frequency sweeps performed at different gaps. Storage modulus $G'$ and loss modulus $G''$ as a function of frequency are shown. During the first hour after the preparation of the monolayer, both moduli are seen to increase by a factor of two (black dots and grey circles). Subsequently increasing the gap from 6 to 22 $\mu$m decreases both moduli.

Figure A.2. Effect of changing the separation between the plates on stress relaxation experiments. Top: imposed strain $\gamma$ as a function of time. A series of steps $\gamma$: 0% $\rightarrow$ 20% $\rightarrow$ 100% is performed at different gap thickness values. Bottom: at 4 $\mu$m, the monolayer appears very stiff. Even though the data is noisy, an increase in stress can be observed after about 100 s. At larger gaps the stresses are about one order of magnitude lower and the data is much cleaner.
way. The stress is seen to increase about 100 s after applying the step strain. At 4 μm, the cells are strongly compressed and the nucleus too is expected to be under compression.

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