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Active microrheology with optical tweezers: a versatile tool to investigate anisotropies in intermediate filament networks

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Abstract

Mechanical properties of cells are determined by the cytoskeleton and especially by intermediate filaments (IFs). To measure the contribution of IFs to the mechanics of the cytoskeleton, we determine the shear moduli of in vitro assembled IF networks consisting of keratin 8/18 and MgCl₂, serving as a crosslinker. In this study we want to present a new method, a combination of active and passive microrheology, to characterize these networks. We also show the applicability of the new method and discuss new findings on the organization and force transmission in keratin networks gained by the new method. We trap and move embedded polystyrene particles with an optical tweezers setup in an oscillatory manner. The amplitude response of the trapped particle is measured and evaluated with a lock-in approach in order to suppress random motions. With this technique we determine the degree of isotropy of the assembled network and sense preferred directions due to inhomogeneities of the network. Furthermore, we show that we can deliberately create anisotropic networks by adjusting the assembly process and chamber geometry. To determine whether there are local network anisotropies in a globally isotropic network, we altered the evaluation method and included the motion of embedded particles in the vicinity of the trapped one. The correlations of the observed motions enable us to map local network anisotropies. Finally, we compare mechanical properties determined from passive with ones from active microrheology. We find the networks measured with the active technique to be approximately 20% more compliant than the ones from passive measurements.

Keywords: keratin, active microrheology, optical trap, intermediate filament, lock-in technique, anisotropy

(Some figures may appear in colour only in the online journal)
of networks by means of a two particle correlation function were published by Crocker et al and Weeks et al [10, 11]. These are all feasible techniques for passive microrheology. However, we want to focus on the challenge of assessing inhomogeneities and force propagation by means of active microrheology.

It is possible to determine the viscoelastic properties by probing the sample with optical tweezers [12, 13]. For active microrheology an external force of distinct quantity and direction is necessary. This force is often applied on the embedded particles using optical tweezers and is in the range of pico Newtons [14–16]. To identify weak local anisotropies in polymer networks, we altered the method of multiple bead active microrheology as follows. One particle is trapped and moved in different predefined directions while the surrounding particles serve as indicators for the motion of the network [17]. This is a very relevant issue since lots of biological materials like cells are supposed to transmit forces in an anisotropic manner due to the nonlinear nature of their interactions. Such anisotropies may modify the real mechanical properties observed by other means. To determine the contribution of intermediate filaments to the total cell stiffness, we already investigated the elastic properties in both in vitro assembled keratin 8/18 networks [18] and extracted pancreatic cancer cells [19]. Using single particle active microrheology, we show that varying the speed of injection of the assembling network into the measurement chamber introduces an anisotropy of the network over the whole sample. Applying a lock-in method we are able to determine the correlated movement on top of the Brownian motion of particles in the vicinity of the excited one. The calculated complex shear modulus is then compared to the conventionally determined one from passive microrheology.

The scope of this paper is to present the above mentioned new method for the determination of anisotropies and direction dependent force transmission as well as proof its applicability on bio-polymeric networks. Also the important issue of appropriate sample preparation in order to avoid biased results is discussed. In the last section of the article the findings obtained with the new method on in vitro assembled keratin networks are discussed and set into context with other publications investigating mechanical properties of cytoskeletal networks.

2. Materials and methods

Human keratin 8 and 18 proteins are isolated and purified as described by Herrmann et al [20]. Afterwards, they are stored at −80 °C in 8 M Urea. Before use they have to be dialyzed into 2 mM Tris-HCl buffer, pH 9.0, where keratin 8/18 is present as tetrameric complexes [21]. Polystyrene particles of 1 μm diameter (Thermo Scientific) are added to the solution to enable microrheology measurements. For assembly activation, 20 mM Tris-HCl buffer, pH 7.0, is added in a volume ratio of 1 : 1. All samples are assembled with a keratin 8/18 concentration of 0.5 g l⁻¹ with 0.25 mM MgCl₂ as a cross linker at room temperature. At least 30 min delay is used to let network properties settle to stable values. The mesh size of the network with the given concentration can be estimated with 435 nm according to Pawelzyk et al [22]. Together with the particle size of 1 μm this ensures proper trapping of the particles inside the network.

The data for passive microrheology is acquired using a custom-made inverted, high resolution and fast recording video microscope. The microscope is equipped with a high-speed camera (Imaging Solutions MotionPro X4, frame-rate up to 5000 Hz at a resolution of 512 × 512 px²) and a high numerical aperture objective (Nikon CFI APO TIRF 100 × , NA = 1.49). From the recorded pictures the position of the particle is extracted via a tracking algorithm in MathWork’s MATLAB [23] leading to an accuracy of approximately 7 nm. For passive microrheology multiple particles are tracked at the same time and by center of mass correction together with the correction factor from Rowlands et al [24] system vibrations and drifts are removed from the data. From these data the MSD can be calculated as $\langle \Delta r^2(1/\omega) \rangle$. The calculation of the complex shear modulus $G'(\omega)$ with $G'(\omega) = k_B T \alpha \omega (\Delta r^2(1/\omega)) \Gamma(1 + \alpha) / \alpha$ the frequency dependent power law exponent, $T$ the temperature and $k_B$ Boltzmann’s constant, follows the procedure described by Mason et al [5]. The real part of $G'$ is the storage, the imaginary part the loss modulus. This method of passive microrheology is quite common and used in this paper to have a method beyond doubt to compare with the newly developed one.

For active microrheology, the setup of passive microrheology is combined with an optical tweezers setup used in different operation modes. The trapping laser beam (coherent compass 1064-500), after passing beam-optimizing optics, can be displaced in two lateral directions by two acousto-optical-deflectors (AOD, AA Opto-Electronic DTSXY-400) which are controlled via frequency generators (Stanford Research Systems, DS345). All processes are triggered, recorded and synchronized by a self-written MATLAB program controlling the camera via USB and all analog signals via the data acquisition module KUBS-3100 (Keithley). The motion of the particle is detected and analyzed with the camera. Prior to measurements the stiffness of the trap is determined with a modification of the algorithm presented in [25] and adjusted to 9.8 pN μm⁻¹.

2.1. Sample preparation and geometry

All samples are assembled in a square sample chamber as displayed in figure 1(a). Keratin is assembled between the microscope slide and the cover slip. The PDMS rings serve as spacers for the chamber with a height of approximately 1 mm and the sample volume is determined by the inner ring (length of 10 mm) to be approximately 100 μl. The assembly solution is injected through one of the inlet-holes, so that the network components spread in the chamber expulsing the air through the other inlet. Since the assembly process starts immediately after mixing the keratins with assembly buffer, an isotropic sample requires fast insertion of the mixture into the sample measurement chamber. In figure 1(b) a schematic of this process is shown. The arrow denotes the flow direction during
insertion of solution. The assembly and thus the direction of the filaments is isotropic over the whole sample. If mixing and pipetting is done too slowly, the partially assembled network is oriented by the afterflowing liquid in the direction of inflow (figure 1(c)). To get a homogeneous network, flowrates of \(100 \mu l s^{-1}\) are used while for the directional assembly the rate was approximately \(10 \mu l s^{-1}\). All measurements are done in an area of approximately \(2 \times 2 \text{ mm}^2\) in the middle of the chamber at a height of approximately \(10 \mu m\) above the cover slip. When more than one particle is measured, care was taken that the \(z\)-positions of the different particles do not differ more than \(1 \mu m\) from each other. This allows us to do all calculations only in the \(x-y\)-plane and neglect the \(z\)-component without making severe approximations.

### 2.2. Single particle active microrheology

The method of single particle active microrheology is used to investigate whether the used preparation technique has an influence on the measured inhomogeneity of the network. Therefore, one particle is trapped by the laser and excited in \(0^\circ, 45^\circ, 90^\circ\) and \(135^\circ\) direction. Thereby the \(x\)-axis (\(0^\circ\)) represents the horizontal, the \(y\)-axis (\(90^\circ\)) the vertical direction compared with picture (b) and (c) in figure 1; hence the inlets are placed on the diagonal (\(45^\circ\) and \(135^\circ\)). The excitation is sinusoidal with a frequency of \(10\) Hz and an amplitude of \(125 \text{ nm}\) in the \(x-y\)-plane and controlled by the AODs. The motion of the particle is captured with the camera at a frame rate of \(1000\) Hz, with \(100\) measured positions per period. To determine the amplitude of the motion, a lock-in technique is applied [17]. The amplitude of the first harmonic of the particle’s motion is isolated computationally by a MATLAB algorithm. The advantage of this method is that the Brownian motion of the particle as well as mechanical vibrations of the system are suppressed since the lock-in algorithm restricts the detected motions to a given

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**Figure 1.** Sample chamber and assembly geometry. (a) Scheme of the sample chamber. Keratin is assembled between the cover slip and the microscope slide with the two insertion holes. The PDMS squares serve as distance holder. (b) Schematic of a fast assembled network with no directionality. The arrow shows the direction of flow. (c) Schematic of a slowly assembled network, inserted through the lower left hole.

**Figure 2.** Measurement of the local anisotropy by single particle active microrheology in a crosslinked keratin 8/18 network. The directionality was introduced deliberately by the sample geometry and a slow mixing of the assembly components. The \(0^\circ\) and \(90^\circ\) directions show a significant difference compared to the \(45^\circ\) and \(135^\circ\) directions. The boxes denote a 25%–50%–75% range of the data and the whiskers a 10%–90% range. The dot gives the mean value.
frequency (10 Hz). The ratio of the measured amplitude and the applied excitation is calculated. Prior, the excitation amplitude is calibrated in water measurements.

2.3. Multiple particle active microrheology

Multiple particle active microrheology is applied to determine the mechanical properties at an intermediate scale (3 μm–20 μm) within the network and to detect intrinsic anisotropies. This approach allows not only to probe the network in the direct vicinity of the trapped and excited particle but also to identify possible force chains or directions along which the excitation of the particle is preferably transmitted to other parts of the network. Therefore the motion of embedded particles in the surrounding are also recorded and related to the motion of the trapped one. Hence the assembly of the isotropically crosslinked network is now done in a fast way to avoid anisotropy on a large scale. As before, one particle is trapped (later referred to as reference particle) and excited with a 10 Hz and 125 nm amplitude sine in the x–y-plane in 9 different directions (20° increments). Due to the symmetry of the excitation an angular range of 180° covers all possible excitation directions. The nomenclature and orientation of the coordinate systems and excitation angles is depicted in figure 3. The camera captures both the reference particle and the motion of the surrounding (response) particles in the network. Again the data is analyzed with the lock-in algorithm. To determine the local anisotropies the response particles’ amplitude is compared to the one of the reference particle. For measurements concerning mechanical properties at intermediate scales, the amplitude of the response particle is evaluated with respect to the distance to the reference particle.

3. Results and discussion

3.1. Global anisotropy

For the demonstration of the ability to produce anisotropic keratin 8/18 networks, the components are mixed slowly and active single particle measurements are performed as described in the methods section ‘single particle active microrheology’. Figure 2 shows the values of measurements from 11 different samples, with 43 different measurement areas and a total of 143 particles. The particles are excited with an amplitude of 125 nm in 4 different directions due to sample chamber geometry. The ratio of the detected particle amplitude and the applied displacement amplitude of the laser is plotted over the excitation angle (i.e. Lock-In Amplitude/Excitation Amplitude).

It is evident that in figure 2 the ratio of the amplitudes in the 0° (x-) and 90° (y-) direction are significantly higher than in the 45° and 135° direction. This means that the particle can follow the excitation in x- and y-direction almost completely, while for the 45° inclined directions the particle moves not even half the distance. This effect can not be seen after fast mixing of the keratin components.

This lets us conclude that the network is stiffer in the direction of the flow and perpendicular to it than in a direction inclined 45° to the flow. The reason is the predominant direction during the assembly process. We assume that in the direction of the flow and perpendicular to it the prestress of the filaments is higher than in the other directions. This reduces the excitation amplitude since the particle has to stretch the network from the beginning of the motion while in the other direction the filaments have to be straightened prior to stretching. The influence of prestress on the Young’s modulus has been investigated by Kreplak et al [26]. The presence of extrinsically induced prestress has some implication for measurements intending to determine global mechanical properties of the network. By averaging all excitation values in order to get an idea of the networks’ stiffness, assuming a constant stiffness over the whole sample, one would get an intermediate value with a high statistical variance. For future measurements it is therefore necessary to verify whether the assembly method leads to long-range isotropic or anisotropic networks.

3.2. Local anisotropy

After determination of the global anisotropy over sample areas larger than 4 mm², it is also interesting to investigate whether a globally isotropic sample provides anisotropies on a local scale. Doing so, it is not only sufficient to have a look at the reference particle as in passive microrheology since it only tests its direct vicinity. It is better to consider the influence of the excited particle on a response particle being located several micrometers apart. This enables us to study the propagation of the excitation and hence the force along the connection line between reference and response particle transmitted via the network. For the measurement the reference particle is excited with the laser in a sinusoidal manner with a frequency of 10 Hz and an amplitude of 125 nm in 9 different directions. In the heatmaps in figures 3(b)–(d), this particle is located in the origin. The excitation of the network with the reference particle displaces the response particles which are distributed randomly over the sample, serving as a marker for the displacements. The excitation direction is rotated subsequently by 20 degrees. So the angle between the response particles and the excitation direction changes with every measurement and therefore the coordinate system of the sample as well (sketched in figure 3(a)). This coordinate system of the sample is denoted with x and y and the coordinate system for the representation in the color maps (b)–(d) of figure 3 is the one with the primes. The x’ axis is aligned parallel to the excitation direction and the angle is measured against the y’ direction. When the excitation direction changes, the coordinate system is also rotated, here denoted with the doubled prime axes. This enables us to measure and represent angles from 0° to 180°. For the color maps the ratio of amplitudes from response and reference particle is calculated and normalized to the appropriate reference particle motion. All dots with the same distance from the origin belong to the same particle measured under different angles. To get from discrete measurement points to a continuous map, linear interpolation is used. The representation is chosen in a way to be consistent with other illustrations e.g. in [27]. Figures 3(b)–(d) are representative illustrations from three different samples.

For all of the measurements the excitation in average decreases with increasing distance of the response particle
to the excitation. This is in good accordance with elasticity theory and was predicted for isotropic, homogeneous samples by Thomson [28]. A typical result for a homogeneous and isotropic network is shown in figure 3(b). The excitation decreases with increasing distance and is approximately isotropic for the first 6 μm indicated by the two innermost response particles. However, for larger distances from the reference particle an anisotropy is visible. The amplitude between 75° and 120° is significantly higher than the amplitude for smaller and bigger angles. This indicates that the excitation is better transmitted in the direction of excitation than in the direction perpendicular, which is in good agreement with the expectation of a homogeneous, isotropic, viscoelastic material [28]. The part of the network in the directions of excitation senses tensile and compressive stresses while for the part in the direction perpendicular to the excitation shear stress is predominant.

In figure 3(c) the ‘equivocolor surfaces’ appear almost elliptical with the major axis perpendicular to the excitation direction, meaning that the excitation is better transferred in a transverse manner. This is counter intuitive and in contrast to the results shown in figure 3(b). One possible explanation is an anisotropy of the network between reference and response particles.

In contrast to the ‘smoothly’ distributed anisotropies of figures 3(b) and (c), in (d) there are prominent changes in the amplitudes within a few degrees/micrometers. In the 30° direction the oscillation is significantly larger than in the surroundings, however in the 130° direction the excitation seems to be transmitted less than in average. Since the particles at 4.5 μm and 5 μm are spatially separated, the 30° and 130° directions correspond to different angles when regarded in the same sample coordinate system. One possible explanation for this behavior is the occurrence of so called force chains [29], here manifested by an enhanced propagation of the excitation in 30° direction. The preferred transmission due to prestressed or tightened filaments can be excluded since the coordinate system is rotated with every measurement as shown in figure 3(a). Furthermore, tightened filaments would also not explain the decrease in the response amplitude in the 130° direction.

3.3. Comparing active and passive results

To compare our results from active multiparticle measurements with previously performed passive microrheology measurements, we need to extract the pure Brownian motion of the response particles. To do so, we calculate the periodical part of the response particle motion with the help of the previously described lock-in amplitude. The MSD of this motion is subtracted from the MSD of the overall motion of the particle. The resulting MSD is the MSD of a bead in a network whose motion is driven by thermal forces only. In order to get the mechanical properties of the surrounding network, the MSD is converted into $G'$ and $G''$ according to [5].

The data for passive microrheology is taken from an identically assembled network. The conversion of the MSD into the shear moduli is done in exactly the same way. In figure 4 the resulting shear moduli, along with the corresponding MSD data in the inset, are shown. The comparison of both MSD’s in the inset yields results at the same order of magnitude and similar shapes. The MSD of the passive measurement is slightly smaller than the one of the reevaluated active one. This means that the particles are able to travel longer distances during the active measurements whereas in the passive case they seem to be more restricted. This can also be seen from the resulting curves for $G'$ and $G''$ in figure 4.

The value of $G'$ from passive measurements is higher than the one from the active method over the whole observed timescale. For the $G''$ curve it is exactly the other way round. Here, the active method delivers slightly higher values. However, the shear modulus of both methods is in the same range. This is a strong hint for the applicability of the methods used in this paper and shows that both techniques are suitable to determine the properties of the network.

Although the results are similar, the difference between them should not be ignored in order to gain further insights into mechanics of the network. Since the networks appear to be more flexible during active measurements, one has to assume that the measurement itself influences the results. During the excitation of the reference particle the network is stretched.
and compressed with forces exceeding those of thermal fluctuations by far, leading to changes in the network structure. A possible explanation for this behavior is the plasticity of the single filaments, meaning that the forces are strong enough and applied for sufficient times so that filaments are irreversibly elongated as stated in [30, 31]. Although both studies are quantitatively not comparable to our measurements—[31] uses higher strain rates and in [30] the filaments adhere to the substrate—they both show qualitatively similar results and lead us to the conclusion that some kind of irreversible process takes place during active measurement. Effects of introduced strain stiffening by the motion of the particle can be excluded since they would increase $G$ significantly as shown for example in [22, 32]. The actively measured network would be stiffer than the one measured passively. The opposite effect of strain thinning might also be a possible explanation for the behavior seen in figure 4.

4. Conclusion

We are able to deliberately produce directional networks by utilizing the geometry of the sample chamber and a slow mixing of the different assembly components to experimentally examine the thereby prepared anisotropies in the network. We show that single particle active microrheology, in combination with a lock-in evaluation method, is capable of identifying global anisotropies in in vitro assembled keratin 8/18 networks by determining the amplitude of the reference particle with respect to the applied excitation of the optical trap. With this method we are able to detect different stiffnesses within the network for distinct directions of excitation. These differences originate from the directional assembly of the network.

With multiple particle active microrheology we can probe the local isotropy of the network by relating the motion of the response particles to the excited reference particle. Depending on the sample, we find regions where the network behaves like an isotropic, homogeneous, viscoelastic medium and regions with strong anisotropies. This sets the stage for further analysis of force transmission in intermediate filament networks.

With the lock-in technique we are able to extract data from active microrheology experiments that can be directly compared to passive measurements. Although being slightly different, the values for MSD and shear modulus are similar for active and passive measurements which is an approval for both methods. A closer look at the differences yields that the actively measured networks are softer by approximately 20% which is in good qualitative agreement with results published in [30, 31]. Nevertheless, further measurements have to be performed to elaborate the biophysical processes behind this softening.

With this in vitro study of crosslinked keratin 8/18 networks we show that active microrheology with an optical tweezers setup is a versatile tool to characterize anisotropies. We demonstrate that mechanical force transmission in IF networks is strongly dependent on the degree of isotropy and can result in both a Thomson-like elastic behavior or in specially confined force chains. This supports the conjecture that besides microtubules and actin also the IF network plays a crucial role in force transmission and mechanical signaling in cells.

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