Molecular and mechanical functions of the intermediate filament protein GFAP
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Towards microrheological probing of the effect of GFAP on cell stiffness

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Abstract

Cells in the human body interact with and respond to their mechanical environment. The cells themselves are an important contributor to the mechanical environment, both through tensile forces and remodelling of the extracellular matrix, and through the cell’s intrinsic material properties. The intrinsic material properties are mainly determined by the cytoskeleton. One of the cytoskeletal elements in astrocytes, the intermediate filament GFAP, is highly regulated throughout development and disease. To investigate the contribution of GFAP to the stiffness of the cell, we used an intermediate filament network (IFN) extraction method to study the effect of recombinantly expressed GFAP isoforms (GFAPα and GFAPδ) on the mechanical properties of the astrocytoma IFN by multiple particle tracking microrheology (MPTM). We developed a robust IFN extraction method to obtain cell-derived skeletonized networks, a useful technique to study biological influences on network mechanics. The analysis revealed that the stiffness of the IFN is on the edge of what can be reliably measured by MPTM, but by comparing the MSD(τ=10) between conditions, a reduced mobility was found when expressing GFAP isoforms GFAPα and GFAPδ in comparison to control.

Introduction

Cells in the human body interact with and respond to their environment. The mechanical environment influences cell migration, motility, proliferation and differentiation. One of the main players in creating this environment and determining its mechanical properties are the cells themselves. They do this by producing and remodelling the extracellular matrix, and by exerting tensile forces using their actomyosin network (Jansen et al., 2015).

The mechanical properties of the cells themselves are important in cell-cell contact situations, and when cells are migrating through a tissue or extravasating from specific tissue boundaries, in the case of e.g. immune response, cancer metastasis, or angiogenesis (Friedl and Wolf, 2010). The cell’s mechanical properties are mainly determined by its cytoskeleton, consisting of actin filaments, microtubules, and intermediate filaments (Huber et al., 2015). Actin filaments and microtubules both provide the cell with structure but are also important for force generation in cell migration and intracellular transport, whereas intermediate filaments are emerging as an integrating hub of cell mechanics and signalling, as described in Chapter 1.

The brain is intuitively not considered the most mechanical of organs, but mechanics also play an important role in this tissue (Tyler, 2012). It is an organ with a highly organised structure. The grey matter contains cell bodies, various
specialized domains and nodes traditionally identified histologically by the difference in cellular densities, whereas white matter contains axonal bundles and the fatty myelin. Recently, it was shown that forces contribute to a constant flow of interstitial fluid through the brain, which is important to clear the brain of debris (Iliff et al., 2012). Where the neurons are the main communicating units in the brain, the glial cells form a crucial supportive cell type important for homeostasis in physiology. Until the intricate and complex biology of glial cells was discovered, glial cells were considered purely as a mechanical filling keeping the neurons together (γλια is Greek for glue). Now it is recognized that glial cells are not only important for structural support, but also for brain functioning. Thus the glial cells are important contributors to the mechanical properties of the brain (Lu et al., 2006), and both neuronal and glial cells are sensitive to these mechanical properties. This mechanical sensitivity of neural cells has been demonstrated in multiple ways: stiffness of the substrate can direct neural stem cell fate (Keung et al., 2011), the presence of rigid implants in the brain induces reactive gliosis (Moshayedi et al., 2014), neuronal firing can be activated by mechanical cues (Coste et al., 2010), and in the extreme case of traumatic brain injury, mechanical trauma can lead to neurodegeneration, loss of consciousness, disability or death (Hemphill et al., 2011; Rosenfeld et al., 2012).

Astrocytes, a glial cell type, have their own unique cytoskeleton due to the presence of an astrocyte-specific intermediate filament (IF): Glial Fibrillary Acidic Protein (GFAP). GFAP forms a network together with the other IFs expressed in astrocytes, i.e. vimentin, nestin, and synemin (Middeldorp and Hol, 2011). Astrocytes increase the production of GFAP when they are faced with a range of pathological conditions, but no clear-cut function has been attributed to the protein yet. We were interested in the effect of increased contents of GFAP on the mechanics of the cell, as a change in these mechanics can have functional consequences for the astrocytes themselves as well as the brain cells around them. GFAP has been shown to affect the mechanics of cells, but contradicting results have been described. In one study, in vivo retinal ischemia induced gliosis in Müller cells, and an increased stiffness was reported with increasing GFAP expression, whereas another study, using in vitro injury of astrocytes reported a reduction in stiffness with increasing GFAP (Lu et al., 2010; Miller et al., 2009).

Different techniques have been developed to measure the mechanical properties of single cells. These include shear, compression, and tensile tests on whole cells, local nanoindentation by atomic force microscopy (AFM), and local probing of the cytoskeleton by passive microrheology with particle tracking
video microscopy, or by active microrheology with optical or magnetic tweezers microscopy (Waigh, 2005). Interpreting measurements on live cells is complicated by the spatial heterogeneity of cells, presence of multiple interacting cytoskeletal networks, and by out of equilibrium activity. An established technique to investigate the IFN is to extract actin and microtubules while retaining the IF cytoskeleton by making use of its insolubility (Lazarides, 1982). Recently, this approach was applied to keratinocytes to probe the role of keratin network changes under shear stress (Sivaramakrishnan et al., 2008). Here, we adopt the same procedure to glial cells, to probe the mechanics of GFAP. To measure the mechanics of the IFN we implemented the technique of multiple particle tracking microrheology (MPTM). One advantage of MPTM over other techniques like AFM, is that in AFM a probe taps on top of the cell and measures the local mechanics on the boundaries of the network, whereas the probes for MPTM are dispersed throughout the network, and thus can give an indication of the local intracellular mechanics of the network. Due to the spatial heterogeneity of cells, MPTM has often been used in living cells, but due to the cell’s non-equilibrium state the interpretation of particle motions in terms of the generalized Stokes-Einstein relation is difficult (Guo et al., 2014). At thermal equilibrium, the mechanics of the network determines the magnitude and time dependence of the displacements the particles undergo by Brownian forces. In a purely viscous liquid, the particle displacements are related to the viscosity via the Stokes relation. For viscoelastic networks, this relation can be generalized to a relation of the particle displacements to the complex shear modulus of the network surrounding that particle (Mason and Weitz, 1995).

To probe the effect of IFN modulation, we started with living cells, modified the IFN in these cells by overexpression of GFAP isoforms with molecular biological tools, and then extracted the IFN from these cells to measure its mechanical properties. The networks we measure are in equilibrium, due to the permeabilization of the cell. This approach combines the advantage of retaining the architecture of the IFN synthesized by a cell, thus providing a reliable biological model for the network, with the advantage of no active forces being at work, facilitating a quantitative interpretation of the data in terms of rheological properties (Brangwynne et al., 2008). We were able to develop a robust cell extraction method to produce cell-constructed IFNs amenable for mechanical measurements. Although the networks from cells transduced with GFAP did display reduced mobility of encapsulated particles, our study revealed that the MPTM method is not sensitive enough to quantify the mechanical properties of the extracted IFNs. However, this could be addressed in future studies by implementing active microrheological techniques.
Microrheological probing of GFAP in extracted IF networks

Methods

Cell lines

For our experiments we used a human astrocytoma cell lines U251-MG (U251). Since the expression of GFAP in the U251 cell line varies, we first selected subclones of U251, and continued with a clone with a low/undetectable basal expression of GFAP (clone D10). This clone was subsequently transduced with lentiviral vectors containing recombinant-mCherry (control), recombinant human GFAPα-IRES-eGFP (GFAPα⁺) or recombinant human GFAPδ-IRES-mCherry (GFAPδ⁺) as described before (Moeton et al., 2016). These transduced populations were again selected for subclones, to obtain 100% transduced populations (control: clone C5, GFAPα⁺: clone G1, GFAPδ⁺: clone E9). U251 cells were maintained in 1:1 Dulbecco’s modified Eagle medium (DMEM) GlutaMAX high glucose: Ham’s F-10 nutrient mix, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (1% P/S) and 10% (v/v) fetal bovine serum (FBS; all Invitrogen, Bleiswijk NL).

For maintenance, all cells were split twice a week and cultured in uncoated tissue culture plastics at 37°C, under a humidified 5% CO₂ / 95% air atmosphere.

Preparation of IF networks with embedded particles

Glass bottom dishes (#0, Mattek corp) were coated with poly-L-lysine (PLL; Sigma-Aldrich) and laminin (PLL (20µg/ml) at 37°C for 30 minutes (mins) followed by laminin (10 µg/ml) at 37°C for 3 hours. Cells were plated at a density of 25,000 cells/35 mm dish and adhered to the dish over night (o/n). Then yellow/green carboxylate modified microspheres of 0.5 μm diameter (F-8888, Molecular Probes) were sonicated and added to the cells in a concentration of 1.75 * 10^7 particles / ml and incubated with the cells o/n. Cell culture medium was refreshed the next day to wash away the particles that were not internalized by the cells. Other methods using a gene gun or particle delivery system were tested for delivery of particles into the cells (Li et al., 2009), but these procedures were unsuccessful in our hands, due to particle aggregation and/or cell death. The procedure for cell permeabilization and IF skeletonization was adapted from a protocol proposed by Sivaramakrishnan et al (Sivaramakrishnan et al., 2008). Briefly, before microscopy analysis, cells were washed twice with phosphate buffered saline (PBS). Cells were then permeabilized in PPST buffer (100 mM pipes (pH 6.9), 1 mM EGTA, 3.5% (w/v) PEG-35000, 0.4M NaCl, 0.25% triton (v/v)) at room temperature for 10 mins. Permeabilized cells were washed once with buffer G (50 mM MES-NaOH (pH 6.2), 0.1mM CaCl₂, 2 mM MgCl₂, 0.5 mM DTT) and then incubated with human gelsolin, kindly provided by F. Nakamura (Harvard Medical School), in buffer G at 37°C for 20 mins. Skeletonized
IF networks were washed 2x in PBS and fluorescent microspheres were tracked in PBS.

**Particle tracking**

Skeletonized IF networks in PBS were equilibrated on the microscope at 37°C. Fluorescent microspheres were excited using an MDF-GFP filter set (Thorlabs).

MPTM was performed with a 100x oil immersion objective on a Nikon TE microscope. Videos were captured at a frequency of 50 Hz with a CCD camera (Photron), as higher frequencies and shorter shutter times resulted in too low intensity of the fluorescent particles. The pixel size of the CCD with the 100x objective was measured to be 0.195 μm / pixel.

**Post-tracking staining, image correlation and particle annotation**

After tracking the mobility of the particles in the IFNs, the networks were fixed in 4% (w/v) paraformaldehyde in PBS (pH 7.4) for 10 mins. Samples were blocked in SuMi (50mM Tris, 150mM NaCl, 0.25 % gelatin, 0.5% Triton X-100, pH 7.4) for 15 mins. To visualize all networks, also the control samples without GFAP, samples were stained with a primary polyclonal chicken anti-Vimentin antibody (1:2000; Chemicon) diluted in SuMi for 1 hour and with a secondary mix of donkey anti chicken-Cy3 and Phalloidin-488 to confirm solubilization of F-actin. Samples were then stored in Mowiol (Calbiochem). Original tracked cells were relocated and imaged using a 40x immersion objective on a confocal microscope (Nikon). A script was written in ImageJ to overlay the positions of the particles in the video tracking to their positions in the immunofluorescence images based on an image transformation calculated based on the coordinates of two distinguishable particles.

Particles were included in the analysis only if they were manually identified as singlets. Particles were then manually annotated to their region within the cell (outside the cell, in the cellular periphery, in contact with the nucleus, or cytoplasmic; an estimation of these boundaries is shown in Fig. 2D). Since the positional overlay of immunofluorescence and the tracking movie was not always 100%, these manual annotation steps were necessary.

**Analysis of trajectories**

Our particle tracking data was initially analysed with the in-house developed particletracker software, making use of a convenient GUI and based on the Crocker algorithm (Stuhrmann et al., 2012). But as many particles had subpixel displacements, this analysis gave large jumps corresponding to pixel-to-pixel transitions. Therefore, another method was adopted where particle centroids were
determined by a radial symmetry algorithm (Parthasarathy, 2012). This algorithm was reported to be more accurate and less sensitive to a low signal to noise ratio than the Crocker algorithm. The radial symmetry code was implemented into a particle tracking and processing algorithm kindly provided by the Kilfoil group (http://people.umass.edu/kilfoil/). Briefly, the procedure consisted of identifying particle locations by a local maximum of intensity. Then the direction and steepness of the intensity gradient within a given radius around the center pixel were calculated on the intersections between the pixels surrounding this local maximum of intensity (Roberts cross operator). The centroid was then calculated by algebraically finding the point with a minimized distance to the lines parallel to the direction gradients on each pixel corner surrounding the local maximum. This minimization took into account a weighing factor, which consisted of the distance of the pixel corner to an initial estimate of the centroid and the steepness of the gradient (Parthasarathy, 2012). A slight alteration was made to the weighing factor as proposed by Parthasarathy (Equation (Eq.) 1).

\[
w_k = \frac{|\nabla I_k|^2}{d_{k,c}} \tag{Eq. 1}
\]

We used an iteration of the radial symmetry center calculation for the initial estimate of the centroid \(d_{k,c}\) and by making the distance to this estimate a relatively larger contribution to the weighing factor than in the original weighing factor (Eq. 2).

\[
w_k = \frac{|\nabla I_k|}{d_{k,c}} \tag{Eq. 2}
\]

Further processing of the individual particles identified per frame were then linked through a tracking algorithm kindly provided by the Kilfoil group, to retain only those features that were present throughout the entire movie (3119 frames), allowing for gaps of at most 1 frame per particle. The coordinates of the feature in this frame were then interpolated from the preceding and the following frame.

The recordings of the particles displayed a drift. Therefore we decided to smoothen the trajectory using a LOESS (LOcal polynomial regrESSion fitting) algorithm in R software for statistical computing (Cleveland et al., 1992). This algorithm calculates a local regression by weighing the distance of datapoints considered in a tricubic fashion. The amount of datapoints per local area considered are based on the spanning parameter \(\alpha\) (0<\(\alpha<1\), which controls the degree of
smoothing. The trajectory was locally fit to a second degree polynomial, with an $\alpha$ of 0.1 (Fig. 3A-F). After subtracting this smoothened trajectory, a small oscillation with a frequency of 2 Hz was still observed (Fig. 3G). This drift was observed in all samples, but was not corrected for, due to the varying amplitude.

The mean square displacements (MSDs) of the features were calculated by (Eq. 3).\[ \text{MSD}(\tau) = \langle (\Delta r(\tau))^2 \rangle = \langle [r(t+\tau) - r(t)]^2 \rangle \] (Eq. 3)

For $\tau_{\text{max}}$ we used $0.25^\ast t_{\text{max}}^\ast$ to allow for sufficient measured displacements for reliable statistics per $\tau$. The procedure was verified on particles dispersed in a mixture of 50% glycerol in water (with a viscosity of 4.5E-3 Ns/m$^2$ (Cheng, 2008)), and the MSD obtained in this procedure corresponded to the theoretical MSD expected for particles in this mixture.

Statistics

The (further referred to as MSD(\tau=10)) was taken to represent the MSD at the point where all the features had a plateau. Statistics on these values were performed in R software for statistical computing. The MSDs between conditions of interest were tested for normality with a Shapiro-Wilk test. Conditions were compared with a nonparametric Kruskal-Wallis test for difference between conditions, and individual contrasts were performed with Dunn’s post hoc test, corrected by Benjamini Hochberg’s False Discovery Rate.

Results

Intermediate filament network extraction

To reduce variability in IFN expression by our cell lines, the heterogeneous U251 cell line was clonally selected for cells expressing low GFAP, but still expressing vimentin. This clonal line was subsequently transduced with mCherry (control), GFAP$\alpha$ (GFAP$\alpha$) or GFAP$\delta$ (GFAP$\delta$). These transduced cells were again clonally selected to obtain cell lines with 100% transduction efficiency. This resulted in three cell lines with three different IFNs: either only the native IF network (vimentin), with low almost undetectable GFAP (control), a network consisting of both GFAP$\alpha$ and vimentin, or a network with vimentin and GFAP$\delta$, known to induce a collapsed network (Fig. 1A-C). The IFs are known to interact with actin and microtubules, which can affect the mechanics of the network (Huber et al., 2015). Therefore we extracted these two components from the cells, while retaining the IFN to determine only the changes in the IFN mechanics, in analogy to a recent publication where the keratin IFN of keratinocytes was studied (Sivaramakrishnan et al., 2008). To retain the pure
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IFN, the cells were permeabilized by 0.5% triton, to remove all soluble proteins. Microtubules were successfully depolymerized and washed away by this step, but the IFN and actin bundles were retained. In some permeabilizations a condensed microtubule spot positive for staining (anti-acetylated tubulin, T6793, Sigma-Aldrich) was still present in the cell, which is likely the microtubule organizing center (Fig. 1D-E, arrowheads in the figure). The networks were treated with gelsolin, a protein that can cleave actin fibers into smaller pieces, to depolymerize the actin bundles. This treatment resulted in a retained IFN in the case of all three cell lines (Fig. 1F-H). Note that the GFAPδ+ cells in the tracking experiment had a much lower fraction of collapses than the cell line shown in the original generation (Fig. 1C). This may be due to the difference in culturing conditions, since for confirmation of the cell line transduction efficiency different culturing conditions were used as in the experiment (uncoated coverslips vs laminin coated coverslips). Also before permeabilization, GFAPδ+ did not show collapses in cells cultured on laminin coated coverslips, so it is not a permeabilization effect (not shown). For all IFNs extracted, attachment to the glass was very stable, as recording sessions of a single sample would usually take 2-3 hours at 37°C, after which the sample was fixed with 4% PFA. In our experiments no loss of complete cells or networks was observed by the permeabilization procedure (Fig. 1D-F), or during the recording. It is likely that the laminin coating of the glass enhances the stability of cell attachment.

Particle annotation and tracking

The time frame in which particles were endocytosed by the cells was between 10-18 hours (not shown). After incubating the cells with the particles for this time, particle-loaded IFNs were obtained by the extraction described. The particles were then tracked by video imaging at a rate of 50 Hz. To interpret the data obtained from each particle in the context of the local IFN, it was necessary to stain the IFN after the tracking. Since the control cells have only low levels of GFAP, we used a vimentin staining to visualize the networks in all cells and imaged the cells in 3D by confocal microscopy. In our earlier work we have shown that vimentin co-localizes and co-collapses with GFAP (Moeton et al., 2016). The particles that were successfully tracked in the movies were correlated to their corresponding confocal images and annotated to their cellular position. The correlation between video tracking and confocal microscopy was based on two reference particles. This approach did not give a completely correct overlap, possibly due to some displacements of the particles that occurred between tracking and staining. The patterns of particles positioning, however, was reliable enough to correctly identify the tracked particles in the confocal images (Fig. 2A-C). Due to the inaccuracy in assignment of
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Figure 1. Generation of clonal lines and intermediate filament extraction
(A-C) A cell line was derived of the U251 line by clonal selection. This clonal line was subsequently transduced with mCherry, GFAPα or GFAPδ. These cells were again clonally picked to obtain a 100% transduction efficiency and immunostained for GFAP (green). Nuclei were counterstained with Hoechst (blue). In the control cells, a very faint GFAP network is present (A). In the GFAPα+ cells, clear networks of GFAP can be seen extending throughout the cell (B). In the cells transduced with GFAPδ, the GFAP network is collapsed into a juxtanuclear aggregate.
(D-H) To extract the intermediate filament network, the cells were exposed to two subsequent steps to first remove soluble content and then to remove polymerized actin. mCherry cells were fixed by a standard procedure and directly stained or immunostained for nuclei (Hoechst),
position of the particles, the annotation of the position in the local network had to be done manually. To this end, four categories of particles were formed: 1) extracellular: particles outside of the cells (coloured white), 2) cell periphery: cells close by the cell edge, often also based on whether the IFN showed a transition from a more homogeneous network to more aligned fibre-like organization (coloured yellow), 3) nuclear contact: particles on top/below the nucleus or touching the outer edge of the nucleus (coloured blue), 4) center of cytoplasm: particles in the rest of the cellular network (coloured green) (Fig. 2D). For the GFAPδ+ cells contact with an aggregate (which is the collapsed IF network) was a separate category (not shown).

The trajectories found for the different particles within a single movie all displayed similar shapes signifying drift of the setup (usually in the order of 100 nm / 60 s; Fig. 2E). Therefore a drift correction per particle was performed based on a LOESS smoothing algorithm, resulting in displacements of 10-100 nm over 60 seconds that could be subtracted from the trajectory (Fig. 2F).

The trajectories were smoothened using a LOESS algorithm, with different $\alpha$ values tested for drift extraction. An $\alpha$ of 0.1 resulted in smoothing the displacement over longer time scales and was used for drift correction. This drift correction was calculated per individual particle, as calculating this over the ensemble of particles in a movie where one or two particles were more mobile than the rest, would result in artificially enlarged displacements for the immobile particles. An example is shown of a stable particle with smoothened drift extraction (Fig. 3A-C) compared to a mobile particle drift with smoothened drift extraction (Fig. 3D-F). It is clear that the drift in the x-direction is similar for both particles (Fig. 3A&D), but the mobile particle is less affected by the drift in the y-direction (Fig. 3B&E). After this correction another LOESS fit was performed with an $\alpha = 0.01$ on the individual particles. This revealed a periodically oscillating drift of 2 Hz present in the sample, with an
Figure 2. Particle tracking and annotation of particles
Movies were made to track the mobility of fluorescent particles in the cell cultures after o/n incubation with a 100x magnification objective. A frame of a movie (3119 frames, 62.38 s) is shown (A). To be able to assign the location of each particle to a cellular position, IFNs were fixed with PFA, stained for vimentin, and imaged with confocal fluorescence microscopy after tracking the particles (B). The process of finding back the original cells was done by hand, and the cells were imaged in a round petridish, therefore there was a slightly rotated angle in the confocal microscopy
amplitude of ca. 5 nm (Fig. 3G). The trajectories were not corrected for this periodic displacement, as the periodic displacements for particles within the same movie varied in amplitude. Therefore the two options available for correcting, i.e. subtracting the averaged displacement of the ensemble of particles or subtracting individually smoothened displacements with $\alpha = 0.01$, would not improve the data. The periodic displacement is likely imposed by an external force, and the difference in displacements between particles may in principle contain information about the local mechanical properties of the network. The MSDs extracted from two different particles, one immobile (Fig. 3H), the other relative mobile (Fig. 3I), were compared with or without drift correction. The MSDs of both particles were affected by the drift correction. The theoretical line of MSD = 4Dt, representing free 2-dimensional diffusion in water at 315 K, is represented by the dotted line going through the origin of the graph. The difference between this steep curve and the measured MSDs illustrates the highly restricted mobility of the particles (Fig. 3H-I).

A difference in mechanical properties at different locations in the cell has been reported for keratin networks (Sivaramakrishnan et al., 2008). Therefore we first investigated if this was the case in our recordings (Fig. 4A). At first glance the particles displayed a very restricted mobility, but we decided to compare the values of the MSD($\tau=10$) between cellular regions. For highest statistical power, all network recordings of the 0.5 um beads were included, regardless of the nature of the IFN. Four separate locations of particles were included in the analysis: extracellular (Median = 95.63 nm$^2$, N=213, IQR: 51.55-275.40), cellular periphery (Median = 99.86 nm$^2$, N=251, IQR = 60.39-212.00), center of cytoplasm (Median = 86.73 nm$^2$, N=505, IQR = 49.86 – 158.8), and particles making nuclear contact (Median = 78.18 nm$^2$, N=316, IQR = 51.17-155.40). Particles that were assigned to multiple location categories were excluded (e.g. a particle attached to the outside of a cell would be in both the “extracellular” and “periphery” categories). The Shapiro-Wilk test for normality rejected the null hypothesis of the normal distribution of the data points (Figure 2 continued) compared to the particle tracking microscopy. An algorithm was developed in ImageJ to correlate the objects identified in the particle tracking microscopy analysis with the confocal image, based on a comparison between the positions of two recognizable particles in both images. The frame of the tracking camera is outlined with the cyan square, whereas all overlaid particles are encircled and labeled with their identifier from the video analysis (C). All particles that were confirmed to be single particles were manually annotated to their location in the cell based on four main regions: extracellular (white), cellular periphery (yellow), cytoplasmic (green), or nuclear (blue) (D). Sample trajectories were plotted with a rainbow colour code for time, ($t_0$=red, $t_{\text{max}}$=violet) from the various regions in the cell before correction for drift (E) and after correction for drift (F). The plotted area amounts to approximately 1 pixel size (0.194 um$^2$), with the vast majority of the particles displaying subpixel mobility. Scale bars: 20 μm.
measured for the samples, therefore non-parametric tests were performed on the data. The hypothesis that different particle mobility occurs in different cellular locations was tested with a Kruskal-Wallis rank sum test. This test reported a significant difference between the subcellular locations, $H(3)=12.57$, $p = 0.0057$. To identify the groups that significantly differed from other groups, a Dunn’s post hoc test was performed, with a False Discovery Rate correction for multiple testing. This revealed a significant effect of bead location on the MSD($\tau=10$), with beads in contact with the nucleus and beads in the central cytoplasm being less mobile than beads in the periphery (Fig. 4A). Surprisingly, beads at extracellular locations, which were expected to be stuck to the glass, had a bigger spread in MSD($\tau=10$) than the intracellular beads. Further analysis of the data implied this spread in the extracellular beads originated solely from a high mobility of extracellular beads in the GFAP$\alpha^+$ condition compared to the control and GFAP$\delta^+$ conditions (Fig. 4D).

To investigate the effect of the presence of GFAP$\alpha$ or GFAP$\delta$ in the IFN, we compared the mobility of beads that were trapped by the cytoskeletal network and were neither in contact with the nucleus nor at the cell periphery. Therefore the beads in the central cytoplasm location were tested for the effect of IFN composition: Control (Median = 102.60 nm$^2$, $N = 168$, IQR $= 62.58-174.10$), GFAP$\alpha$ (Median = 80.30 nm$^2$, $N = 231$, IQR $= 49.16-171.10$), and GFAP$\delta$ (Median = 68.95 nm$^2$, $N = 106$, IQR $= 40.96-127.60$). The hypothesis of different mobility in different IFNs was tested with a Kruskal-Wallis rank sum test. This test reported a difference between the different networks, $H(2)=15.41$, $p < .001$. To identify which groups differed significantly from the other groups, we performed a Dunn’s post hoc test. This reported a different mobility of particles between all groups in the order of GFAP$\delta$ < GFAP$\alpha$ < control (Fig. 4B).

We expected to observe many complete IFN collapses in the GFAP$\delta$ condition. Surprisingly, the percentage of cells with collapses in the MPTM experiment was a lot lower than the fraction when we initially verified the cell line (38% vs 100%, Fig. 1H vs 1C). Therefore, we were unable to reliably test the effect of collapsed networks on bead mobility. To investigate whether network collapses had an effect on bead mobility, we studied the data for the GFAP$\delta$ samples per cellular location. However, the number of beads in contact with aggregate was low (periphery and collapse: $N=9$, central cytoplasm and collapse: $N = 21$, nucleus and collapse: $N=13$). Therefore we pooled all intracellular beads and tested with Wilcoxon’s rank sum test for a difference between beads in the network (Median = 76.09 nm$^2$, $N = 235$, IQR $= 45.56-129.52$) versus beads at collapses (Median = 121.46 nm$^2$, $N = 43$, IQR $= 54.77-229.57$). A significantly higher mobility was observed for the beads in contact
Figure 3. Extraction of the MSD from the trajectories of particles embedded in IFNs
(A-F) Calculation of the drift of two objects based on a trajectory smoothened with a LOESS local
regression algorithm ($\alpha=0.1$), comparing a relatively static particles (A-C) and a relatively mobile
particle (D-F). Shown is the displacement over time for x (A, D) and y (B, E) and the resulting
trajectory in x and y (C&F), with the trajectory in black and the calculated drift in white. (G) After
correction for this calculated drift per particle, a periodic oscillation of 2 Hz was still observed in
the individual particles when smoothened with a LOESS with $\alpha=0.01$ (black traces) as well as in the
ensemble of the particles (white, mean of all smoothened fits). No correction was performed for this
oscillation. (H-I) The extracted MSD was plotted against the lag time extracted for the uncorrected
and the corrected data, for either the relatively static particle (H) or the relatively mobile particle (I).
The dotted line going through the origin of the graph represents the MSD=4Dt relation expected
for Brownian motion of 0.5 um particles in water at 315K, illustrating that both particles display
highly restricted displacements. For further statistics the MSD(<$\tau$=10s>) (vertical line) was used to
compare the amplitude of motion between conditions. MSD(<$\tau$=10s>) was calculated by averaging
the MSD(<9s<=$\tau$<11s>) (dashed lines), to eliminate the periodic variation found in Fig. 3G.
Figure 4. Analysis of the MSD(τ=10s) as a function of cellular location and IF network composition
(A) The MSD(τ=10s) was determined for all particles meeting the inclusion criteria. All the GFAP isoform conditions were pooled to detect if there was an effect of the location of the particle in the cell on the MSD. Particles that could not unambiguously be assigned to a single category were excluded from analysis.

(B) GFAP network effect on the MSD of particles localized in the cytoplasm. Boxplots show the distribution of the MSD(τ=10s) in a cell with a control network, a GFAPα network, or a GFAPδ network. To probe only particles trapped by the network, and not by the nucleus or the cell edge, only particles classified as cytosolic were taken into account. Cells with a GFAPα or GFAPδ network showed a slightly but significantly reduced MSD(τ=10s).

(C) As the GFAPδ cells were in some cases loaded with a collapse of IFs, we separately plotted the effect of location of a particle in a cell with a GFAPδ network and indicated the effect of the particle being in contact with an aggregate (blue dots in contact with aggregate, red dots not in contact with aggregate, boxplots represent all datapoints).

(D) An overview of the effect of particle location per IFN. It shows a decrease in MSD(τ=10s) for GFAPα+ and GFAPδ+ at the cell periphery and cytoplasm, and an unexpected increase in MSD(τ=10s) for the GFAPα+ extracellular particles in contrast to control and GFAPδ+. 
with a collapse $W_s = 3300, p = 0.016$ (Fig. 4C).

**Discussion**

GFAP is the signature IF of astrocytes and astrocytoma cells. The protein is highly regulated in development and disease, but the effect it has on cell mechanics has not unequivocally been demonstrated. Two studies investigating this in living cells by AFM probing described either increased or decreased stiffness with increasing GFAP expression (Lu et al., 2010; Miller et al., 2009). Here we implemented a different method based on MPTM to study whether a change in IF composition will result in different mechanics of the IFN extracted from cells. Our data indicate increased stiffness of the IFN with increasing GFAP expression, but the method is not sensitive enough for the IFN and should be supplemented with active probing methods to extend the range of sensitivity. Here we will discuss the technical aspects and challenges met in our approach. Finally we will give some suggestions for further investigation into the effect of IFN composition on IFN mechanics.

In this study we used MPTM as a method to investigate the mechanical properties of different compositions of cell-extracted IFNs of astrocytoma cells. This method has been used in various other studies before to investigate cellular mechanics in living cells (Daniels et al., 2010; Guo et al., 2014; Kole et al., 2004) or in vitro constituted gels of biopolymers (Apgar et al., 2000; Aufderhorst-Roberts et al., 2014). Here we used an approach in between *in vitro* and *in vivo* (in cell), by probing networks constructed by cells and subsequently extracted for mechanical tests, as described before for keratin networks (Sivaramakrishnan et al., 2008). This has the advantage that the complexities originating from 1) the many different proteins contained by the cell and 2) the active forces exerted by the cell are greatly reduced or no longer of concern. The first point is extra relevant in the case of IFs since they do not only have a mechanical function in the cytoskeleton, but are emerging as important signaling platforms with interactions with, and regulatory functions for, actin and microtubule networks (Helfand et al., 2011; Ivaska et al., 2007; Jiu et al., 2015; Sakamoto et al., 2013). A recent study by Guo et al gives a good example of the implications of active forces in cellular microrheology (Guo et al., 2014). They compared particle displacements in normal cells to cells where active transport had been blocked by either ATP depletion or by applying an inhibitor of non-muscle myosin II, the main protein responsible for the active forces in cells (Koenderink et al., 2009). In the cells with active transport, particles showed behaviour reminiscent of unhindered Brownian diffusion. However, inhibition of active transport rendered the MSDs nearly time independent, effectively implying elastic behaviour
of the cytosol (Guo et al., 2014). The time independent behaviour we observe in the extracted IFNs seems consistent with this observation of the necessity of active forces for diffusive behaviour in a cytoskeletal network, although we lack of course the microtubules and actin filaments.

The main challenge in the current study was that the displacements of the particles in the IFN networks were small. Although comparable to the trajectory size in the earlier study on keratinocytes, we could not extract viscoelastic behaviour from the data (Sivaramakrishnan et al., 2008). This made the system very sensitive to noise and resulted in difficulties determining the actual trajectories of the particles. In a first approach, centroids were determined using an often used algorithm (Crocker et al., 1996). This method identifies features based on local maxima, meeting the proper criteria expected from the particles under study (e.g. size and brightness). However this method was sensitive to the shifting of a local maximum from one pixel to the next, and this would often result in a calculated displacement larger than the subpixel Brownian motion of the particle. Therefore we switched to a more robust method of particle center determination, based on the calculation of the radial symmetry center (Parthasarathy, 2012). This method is less sensitive to the signal to noise ratio, and in our experience also reduced the jumping behaviour induced by the displacement of the local maximum of a feature. This method indeed reduced the number of artefacts found in particle tracking. With this improved precision of particle localization, displacements remained very small, and were dominated by the drift of the sample (Fig. 2E). After subtraction of the drift, the trajectories of the particles were likely close to the resolution limit of our setup. However, despite these small displacements, differences could still be observed between particles based on location in the cell (Fig. 4A). It is possible that the differences observed between cellular locations are caused by the position of the particles above or below the focal plane. We inspected whether particle intensity, as a measure of out-of-focusness for a particle, had an effect on MSD. Although low particle intensity did result in a narrower distribution of MSD(τ=10), the particles at different cellular locations had a similar distribution across particle intensity (not shown). With the small displacements observed in our experiments, the errors of the measurement become a large contribution of the trajectories measured. For MPTM, there are two main contributions to the error of a measurement, the static error and the dynamic error. The static error depends on the signal to noise ratio of the image of a particle, and determines the effective spatial resolution of the setup, whereas the dynamic error originates from the motion of a particle during the recording of a frame and is shutter time dependent (Savin and Doyle, 2005a).
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Cytoskeletal networks were modelled as a Kelvin-Voigt viscoelastic material, where at short time scales particles display diffusive behavior (MSD scales with power 1), whereas at longer time scales they can reach a plateau phase, where elastic properties dominate. Savin and Doyle demonstrated that especially in the case where the shortest lag time is too short to determine the diffusive behavior, the MSD in the plateau phase will be underestimated (Savin and Doyle, 2005b). With the dataset currently available, the dynamic error cannot be determined, as extensive calibrations are required for this. Therefore we cannot extract the viscoelastic modulus from the measured particles trajectories by the methods that have been developed for MPTM. The static error of our measurement could be extracted from immobile particles. Although initially we intended to determine this based on the displacement of particles attached to the glass, we observed smaller MSDs in the case of cells embedded in the network, especially when in contact with the nucleus (Fig. 4). Also the particles that are located extracellularly are not sticking to the glass directly, as this has been coated with a layer of both PLL and laminin before experiments. Laminin is an extracellular matrix protein that can have its own mechanical properties, depending on the thickness of the laminin layer deposited on the glass slide. Without clearly defined immobile particles, it could be an option to determine the resolution based on the least mobile set of beads, but it is better to make a controlled sample with a larger number of beads fixed to a surface and measure the displacements in such a sample as a control for the static error.

To place our measurements into context, we determined values reported in the literature for the mechanical properties of IFNs. For cells without a vimentin component in the cytoskeleton, an elastic modulus of 5 Pa has been determined at 1 Hz by active microrheology, which increased to 9 Pa when a vimentin network was expressed (Guo et al., 2013). This is still in the context of a live cell and thus in a composite network, but also by using \textit{in vitro} constituted networks, elastic moduli of 1-10 Pa have been reported for vimentin networks (Lin et al., 2010a, 2010b; Pawelzyk et al., 2014). If the IFN extracted from our cells has an elastic modulus in the range of 1-10 Pa, then this is indeed above the upper limit of MPTM, as this is around 1 Pa. Methods more suited to probe stiffer networks include active microrheology techniques, such as optical tweezer microrheology or magnetic microrheology (Waigh, 2005).

Apart from this inherent sensitivity limitation of the technique, there were two more limitations to our assay. Although we tried to control the particle-network interaction in a cell-derived network to the best of our abilities, two important factors were not controlled. On the one hand, the exact composition of the network,
both in terms of specific IF protein contributions, as well as the insoluble IF-binding proteins that are not released upon permeabilization and washing, are not known. This is clearly illustrated by the fact that the network is staying attached to the coverslip, implying that integrins and other focal adhesion proteins anchoring the network to the extracellular laminin are not washed away. On the other hand the specific interactions of the particles with the network are not controlled. The particles have a surface chemistry of carboxylate groups, which has a lower interaction with proteins than a polystyrene surface, although not as low as particles with a polyethylene glycol (PEG) surface chemistry (Valentine et al., 2004). A test was performed with PEGylated particles, but cells did not endocytose these particles. We expected the carboxylate particles to be endocytosed, and released from the endocytic vesicles by the triton treatment, after which they would be trapped in the IF network. Tests were performed to confirm this through staining for LAMP1 (a marker for late endosomes and lysosomes), but the strong fluorescent signal of the particles and subsequent bleed-through in all other microscopy channels made it difficult to interpret these micrographs (not shown). Although the particles that we observe in the tracking experiment are clearly trapped, it is not clear if this is due to specific interactions with or capture by the IFN or encapsulation by proteins in the endosome that may be linked to the IFs. Particles of 0.2 μm and 1.0 μm size showed a similar limited mobility as described for the 0.5 μm particles (not shown). The fraction of particles that showed displacements larger than pixel size (195 nm) was <1%, and most of these particles were no longer present after fixation and staining.

Although our results cannot be interpreted quantitatively, we made a semi-quantitative comparison between the tested conditions, by using the MSD(τ=10) as a measure for the stiffness of the network. We observed an effect of the intracellular localization of the particles on the apparent MSD, which is in line with earlier findings on the mechanics of keratinocytes, where particles in the periphery of the cell were more mobile than particles near the nucleus. In the keratinocytes this was attributed to the IF network around the nucleus being more dense with smaller mesh sizes, whereas at the periphery of the cell it is more sparse (Sivaramakrishnan et al., 2008). Although our confocal images do not have a high enough spatial resolution to determine mesh size, the distribution of the network can be seen to be heterogeneous (Fig. 2B). The decreased MSD of networks extracted from GFAPα and GFAPδ transduced cells as compared to control networks is intuitive, when considering all IFs to contribute to the overall network stiffness (Lin et al., 2010a). The absence of collapses in the GFAPδ+ samples was surprising, but an effect of GFAPδ on IFN geometry could be seen, with vimentin immunoreactivity at the periphery
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of the cell showing less bundles (not shown) and some collapses occurring (Fig. 1H, arrows).

**Future directions**

The goal of this study was to investigate the effect of a difference in IFNs with different compositions on network stiffness. In the future this method can be relevant to study IFNs from astrocytes that have been exposed to inflammatory, pathological or pharmacological stimuli to identify the mechanical effect of astrocyte reactivity or neural stem cell differentiation. Although the passive MPTM method is not sensitive enough to reliably describe the mechanical behaviour of the elastic IFN, the goal of characterizing IFN mechanics is an interesting one, as different mechanical properties have been described for different IFNs, attributed to the interactions different IF subunits have with one another (Pawelzyk et al., 2014). Therefore adding GFAPα or GFAPδ to a vimentin-dominated network, can have interesting effects on the total IFN mechanics. Although currently investigations are being done into composite networks of two-component systems containing either actin, microtubules or IFs to identify emerging properties (Jensen et al., 2014; Lin et al., 2011; Pelletier et al., 2009), the composite networks consisting of different IF proteins with their specific side arms and backbone charges, can also deliver interesting insights into network formation and material properties (Kornreich et al., 2015). The method used in this paper to extract IFNs from cells, and potentially composite networks of IFs and actin stress fibers if the gelsolin treatment is skipped, is a robust method for further studies. On the one hand, it offers perspectives for characterizing the network properties of IFNs or actin IF composite networks, polymerized and geometry defined in a cell, allowing for an in-between model for *in vitro* (test-tube network) and *in vivo* (living cell). Although, as mentioned before, the particle interactions should be characterized, by either making the particle non-sticking, or targeting it specifically to a component of interest (Valentine et al., 2004; Wong et al., 2014). For embedding the particles in the network a different technique may be required, which circumvents the endocytosis machinery of the cell. Although this is usually done by microinjection, this is a laborious process, and not very suitable for obtaining large statistics across many cells rapidly. A novel method developed to transduce cells with protein via osmosis-induced macropinocytosis may be an interesting candidate to transduce cells with tracer particles (D’Astolfo et al., 2015). To probe the skeletonized networks obtained in this way, the method should then be switched to the active microrheology procedures, to gain a broader sensitivity.
Chapter 6

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