Origin of slow stress relaxation in the cytoskeleton - Supplementary Information

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Protein purification and network formation.

The actin crosslinker human α-actinin 4 (ACTN4) was purified as described in reference [1]: Rosetta E. Coli cells were transformed to express recombinant crosslinkers with a 6xhis-tag. Induction was performed with 500 μM isopropyl β-D-1-thiogalactopyranoside for eight hours at 25 ^{o}C. After centrifugation at 6000 g for 15 minutes, cells were resuspended in 20 mM NaCl, 5 mg/ml lysozyme and 20 mM Hepes, pH 7.8. The cells were lysed by a freeze-thaw cycle, and centrifuged at 20,000 g for 30 min. The recombinant protein was purified from the supernatant using a QIAGEN nickel column. Next, the column was washed with 20-bed columns of 500 mM NaCl, 25 mM imidazole, and 20 mM Hepes, pH 7.8. The recombinant proteins were eluted with 10-bed volumes of 500 mM NaCl, 500 mM imidazole, and 20 mM Hepes, pH 7.8. The proteins were concentrated using a Centricon filtration device (Millipore) and purified by gel filtration in 150 mM NaCl, 20 mM Hepes pH 7.8, and 10 mM dithiothreitol (DTT) using an AKTA purifier (GE Healthcare) and a Sephadex 200 column.

Actin was purified from rabbit psoas skeletal muscle as described in reference [2] and stored at $-80\,^o$C in G-buffer (2 mM tris-hydrochloride pH 8.0, 0.2 mM disodium adenosine triphosphate, 0.2 mM calcium chloride, 0.2 mM dithiothreitol) to prevent polymerization. We used a concentration of 48 μM (2 mg/ml) for all our experiments and actin was polymerized in a buffer consisting of 50 mM KCl, 20 mM imidazole pH 7.4, 2 mM $MgCl_2$, 1 mM DTT and 0.5 mM MgATP (F-buffer). Unless otherwise mentioned, we used a crosslinker concentration of 0.48 μM to obtain a molar ratio of 1/100 crosslinker/actin and on average about 1 crosslinker per 0.5 μm of actin filament; under these conditions, networks are unbundled and isotropic as verified by confocal fluorescence microscopy [Fig. S4]. All chemicals were bought from Sigma Aldrich unless otherwise mentioned.

Rheology

Rheology was performed using a stress-controlled Kinexus Malvern Pro rheometer with a stainless steel 20 mm radius cone plate geometry with a 1^o degree angle. We loaded 40 μl of the ACTN4 crosslinked actin networks directly after mixing the proteins into the polymerization buffer. A thin layer of Fluka mineral oil Type A was added around the geometry to prevent evaporation, and the sample was closed off with a hood to minimize effects of air flow. Polymerization of the network was followed by applying a small oscillatory shear with a strain amplitude of 0.5 % and a frequency of 0.5 Hz for 2h. Next, a frequency sweep was performed between $0.01-10$ Hz, using 10 data points per decade and an amplitude of 0.5 %. Afterwards, 2D stress/frequency rheology was performed [Fig. S2a] by varying both the prestress ($0.2-8$ Pa) and the frequency ($0.01-10$ Hz) with an amplitude of oscillation that equals 10% of the applied constant stress. Frequencies above 10 Hz could not be measured as inertial effects from the rheometer dominated the rheological response of the actin network at high frequencies. The prestress was increased from 0.2 Pa to 8 Pa with 0.2 Pa intervals. At every step in prestress, a superimposed small amplitude oscillation was applied with a frequency of oscillation that started at a frequency of 10 Hz for every prestress and was decreased to 0.01 Hz in 20 logarithmically spaced steps using 4 cycles per frequency. After reaching 8 Pa, the prestress was decreased with 0.2 Pa intervals, again performing a frequency sweep at every prestress. All experiments were performed at $T = 298$ K.

In the main text, all figures show data from the decreasing sweep as the viscoelastic flow due to the prestress was smaller for the decreasing sweep than for the increasing sweep [Fig. S2b]. However, we show that similar trends are observed in the upward stress/frequency sweep [Fig. S3]. Furthermore, the stress/frequency sweep data in the main text [Fig. 2] and [Fig. 4a,b] only show a representative selection of the experimental data to prevent graphs from becoming crowded, whereas the full data sets are shown in [Fig. S3].

Fitting procedure

We fit the differential storage modulus with Eq. (9) from the main text using a least square fitting algorithm in two steps. In the first step, we fit the data with all parameters (pre-factor, characteristic frequency and an onset stress of stiffening) fixed over the entire stress/frequency range. In the second step, we refine the fit by allowing for a stress-dependent characteristic fre-

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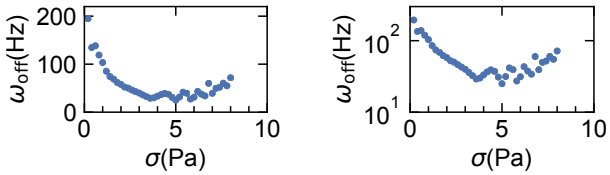
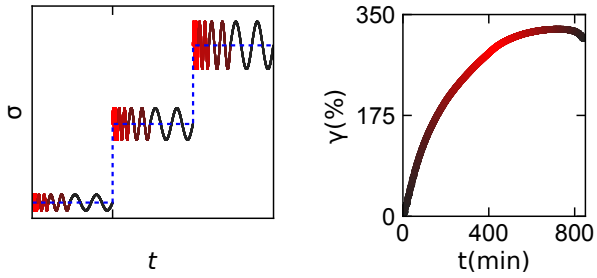


(a) (b)

Figure 1. **Characteristic frequency indicative of the crosslinker unbinding rate as a function of the applied stress.** The differential storage modulus was fit at every prestress with Eq. (9), using the characteristic frequency as a free parameter. Data are shown on a lin-lin (a) and log-lin scale (b). The prefactor and onset stress of stiffening (σ_0, not $\sigma_{0,\mathrm{tr}}$) were fixed at resp. 748 Pa and 11.8 Pa.



(a) (b)

Figure 2. **Stress protocol and creep.** a) Schematic of the 2D stress/frequency protocol. We apply a prestress (dashed blue line) with a superimposed small amplitude oscillation, varying both the frequency and prestress in time. Using this protocol, we measure the differential storage and loss moduli. The frequency is color coded. b) During the 2D stress/frequency sweep, the actin network is under continuous stress and therefore viscoelastically flows. Here we plot the strain as a function of time, with $t = 0$ corresponding to the start of the sweep. The prestress is color coded from 0.1 Pa in black to 8 Pa in red. After approximately 400 min, a full 2D scan is completed and the stress is ramped down to measure the reversibility of the stiffening ($400 - 800$ min).

quency, using the pre-factor and onset stress of stiffening from the first step. Note that the characteristic frequency obtained in this way is larger than the highest probed frequency over the full stress range [Fig. S1]. Therefore, the values should be interpreted with caution as the absolute values are sensitive to the choice of pre-factor and onset stress of stiffening. However, the decrease in characteristic frequency with prestress shown in [Fig. S1] should be robust.

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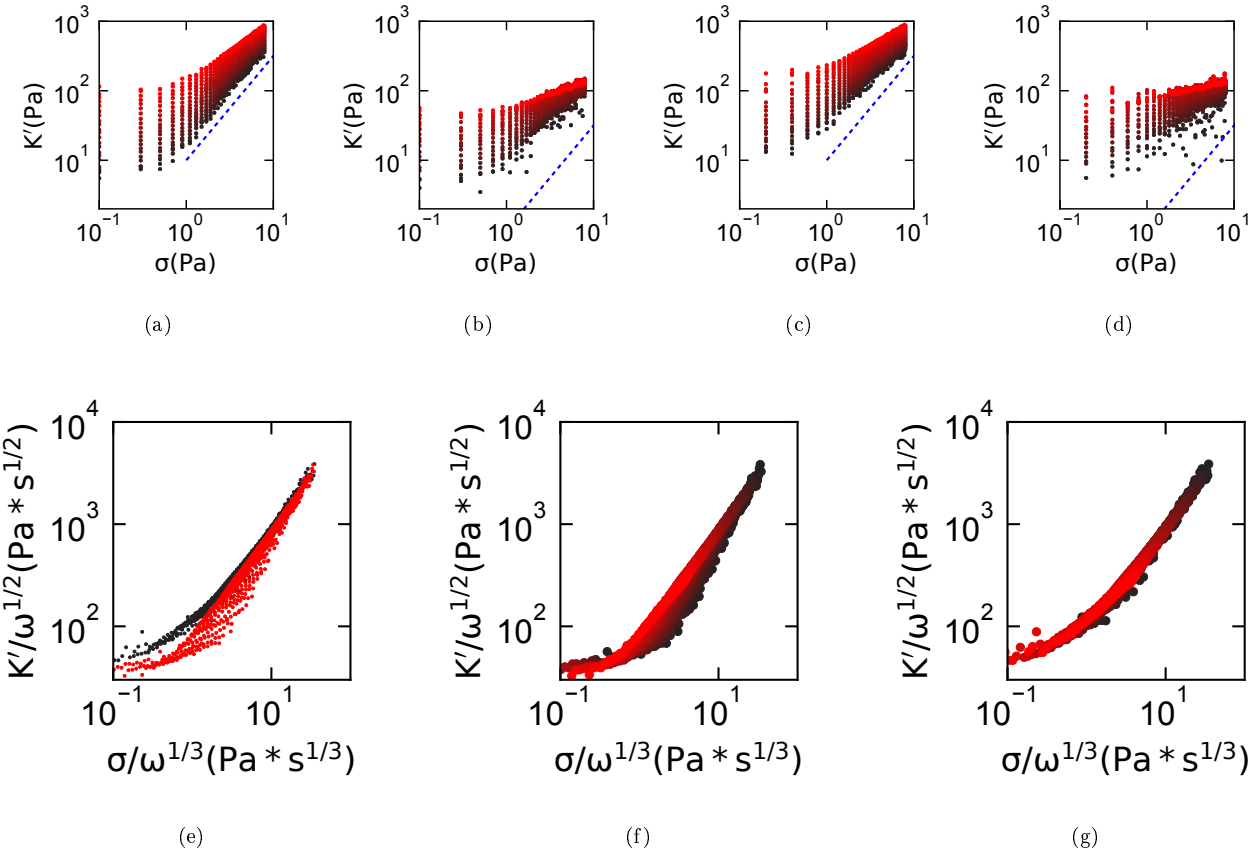

Figure 3. **Comparison of upward and downward 2D stress/frequency sweeps.** The differential storage (a,c) and loss (b,d) moduli for the upward (a,b) and downward (c,d) stress sweep. The dashed line shows the 3/2 power law expected for permanently crosslinked networks. e) Using Eq. (9), we collapse the upward (red) and downward (black) curves - showing that the stiffening and frequency dependence are both insensitive to the sample history. The individual upward (f) and downward (g) curves are color coded as a function of frequency similar to [Fig. 3] from the main text. Note that Fig. S3g is identical to Fig. 3b and only shown again here for ease of comparison.

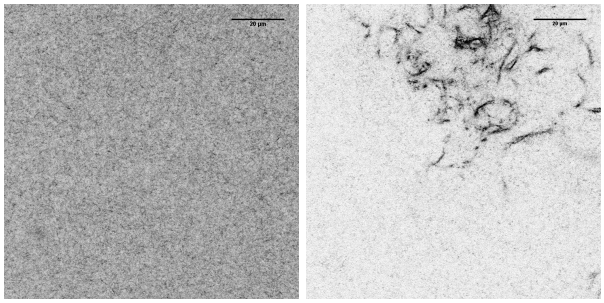

Figure 4. **Confocal fluorescence images of ACTN4 crosslinked actin networks** a) At a 1:100 ACTN4:actin molar ratio, the concentration used for all experiments in this manuscript, an isotropically crosslinked actin network is observed with no structure above the diffraction limit. b) For comparison, actin bundles were observed at a 1:25 ACTN4:actin molar ratio. The color coding was inverted for both images to improve the visual contrast between bundles and background. Scale bars are 20 μm.