**Supplemental Information for**

**Quantifying cytoskeleton dynamics using differential dynamic microscopy**

**Details of sample preparation used to generate data for the first part of the Representative Results section on vimentin networks**

1. **Sample preparation**
	1. Prepare recombinant human wild type vimentin using Escherichia coli (strain TG1) as described in1, using a plasmid gifted by H. Herrmann, German Cancer Research Centre.
	2. Store the protein at -80 °C in a storage buffer (5 mM tris(hydroxymethyl)aminomethane (Tris) pH 7.5, 1 mM dithiothreitol (DTT), 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM ethylene glycol bis (b-aminoethyl ether) N,N’-tetraacetic acid (EGTA)) supplemented with 8 M urea.
	3. Dialyze the protein against vimentin buffer (5 mM Tris-HCl (pH 8.6), 1 mM EDTA, 0.1 mM EGTA, and 1 mM DTT) with stepwise decreasing urea concentrations to refold, the protein as described in1,2 but using a mini-cassette with a molecular weight cut off of 20,000 for dialysis. In the final step, dialyze the protein into vimentin buffer without urea.
	4. Determine the protein concentration by dividing the measured UV absorbance at 280 nm with an extinction coefficient1 of 22450 M-1cm-1 .
	5. Passivate polystyrene beads with diameters of 0.6, 0.8 and 1.1 µm using the protocol described by Jansen et al.3 based on work from Kim et al.4. Briefly, swell the beads in anhydrous (99.8%) toluene to allow F-127 Pluronic molecules, added as a solution of 1% (w/w), to insert, making the bead surface chemically inert. Wash the samples to remove toluene.
	6. Make imaging chambers by placing transparent film stripes between a high precision coverslip (170 ± 5 μm) and a thicker glass slide.
	7. Clean the coverslips with ethanol and passivate with 0.1 mg/mL Poly (L-lysine) polyethylene glycol biotin (PLL-PEG-bio, as PLL (20)-g[3.5]-PEG (2)/PEG (3.4)-Biotin(20%)) to prevent nonspecific interaction of the proteins with the glass surface.
	8. For imaging, prepare a sample mix on ice such that the final concentration of the assembly buffer is 40 mM Piperazine-N, N′-bis (2-ethane sulfonic acid) (PIPES; pH=7.4), 70 mM KCl, 4 mM MgCl2 and 1 mM EGTA. Add DTT and magnesium adenosine triphosphate (ATP) to a final concentration of 1 mM and 0.5 mM, respectively.
	9. Add particles and proteins separately to each of the two independent replicates. Chose the particle volume in such a way that there are 5-20 particles per imaged region of interest (ROI).
	10. Lastly, just before sample loading, add vimentin and resuspend the mixture slowly but thoroughly. Directly after loading, seal the chambers with vacuum grease. Incubate the samples at room temperature for 3-4 h to allow for network polymerization.
2. **Image acquisition**
	1. Take videos using an inverted microscope equipped with a 100x oil objective having a numerical aperture of 1.4 and with a CMOS camera.
	2. Choose the image plane to be at least 20 µm above the surface (marked by the edge of the channel). Chose ROIs as random areas in the middle of the imaging chamber and with approximately 10 to 20 particles, and a minimum of 5 particles.
	3. Acquire videos in brightfield mode, using a ROI with dimensions of 1024 pixels x 1024 pixels. After binning the images at 2 x 2, select a final ROI of 512 pixels x 512 pixels corresponding to 66.56 µm x 66.56 µm. Set the exposure time to 10 ms – resulting in an effective frame rate of 99.9 frames per second.
	4. Make two to three videos of 5000 loops per independent sample without delay between frames.

**Example parameters file for the analysis of data presented in the Representative Results section on vimentin networks**

DataDirectory: 'Z:\\Hannah - vimentin networks\\m05\\'

FileName: '210624\_Vimentin\_m05\_p11\_B\_.nd2'

Metadata:

 pixel\_size: 0.13 # size of pixel in microns

 frame\_rate: 99.92 # frames per second

Analysis\_parameters:

 starting\_frame\_number: 0

 ending\_frame\_number: null

 number\_lagtimes: 60

 first\_lag\_time: 1

 last\_lag\_time: 1000

 crop\_to\_roi: null

 split\_into\_4\_rois: no

 use\_windowing\_function: no

 binning: no

Fitting\_parameters:

 model: 'ISF - Single Exponential - NonErgodic'

 Tau: [1.0, 0.01, 2000]

 StretchingExp: [1.0, 0.4, 1.2]

 Amplitude: [1e3, 1, 1e8]

 Background: [5e4, 0, 1e5]

 NonErgodic: [0.9, 0, 1]

 Good\_q\_range: [13, 80]

 Auto\_update\_good\_q\_range: False

**REFERENCES:**

1. Aufderhorst-Roberts, A., Koenderink, G. H. Stiffening and inelastic fluidization in vimentin intermediate filament networks. *Soft Matter.* **15**, 7127–7136 (2019).

2. Herrmann, H., Kreplak, L., Aebi, U. Isolation, Characterization, and In Vitro Assembly of Intermediate Filaments. *Methods in Cell Biology.* **78**, 3–24 (2004).

3. Jansen, K. A. Extracellular Matrix Mechanics and Implications for Cellular Mechanosensing — Vrije Universiteit Amsterdam. https://research.vu.nl/en/publications/extracellular-matrix-mechanics-and-implications-for-cellular-mech (2016)

4. Kim, A. J., Manoharan, V. N., Crocker, J. C. Swelling-based method for preparing stable, functionalized polymer colloids. *Journal of the American Chemical Society* **127** (6), 1592–1593 (2005).