

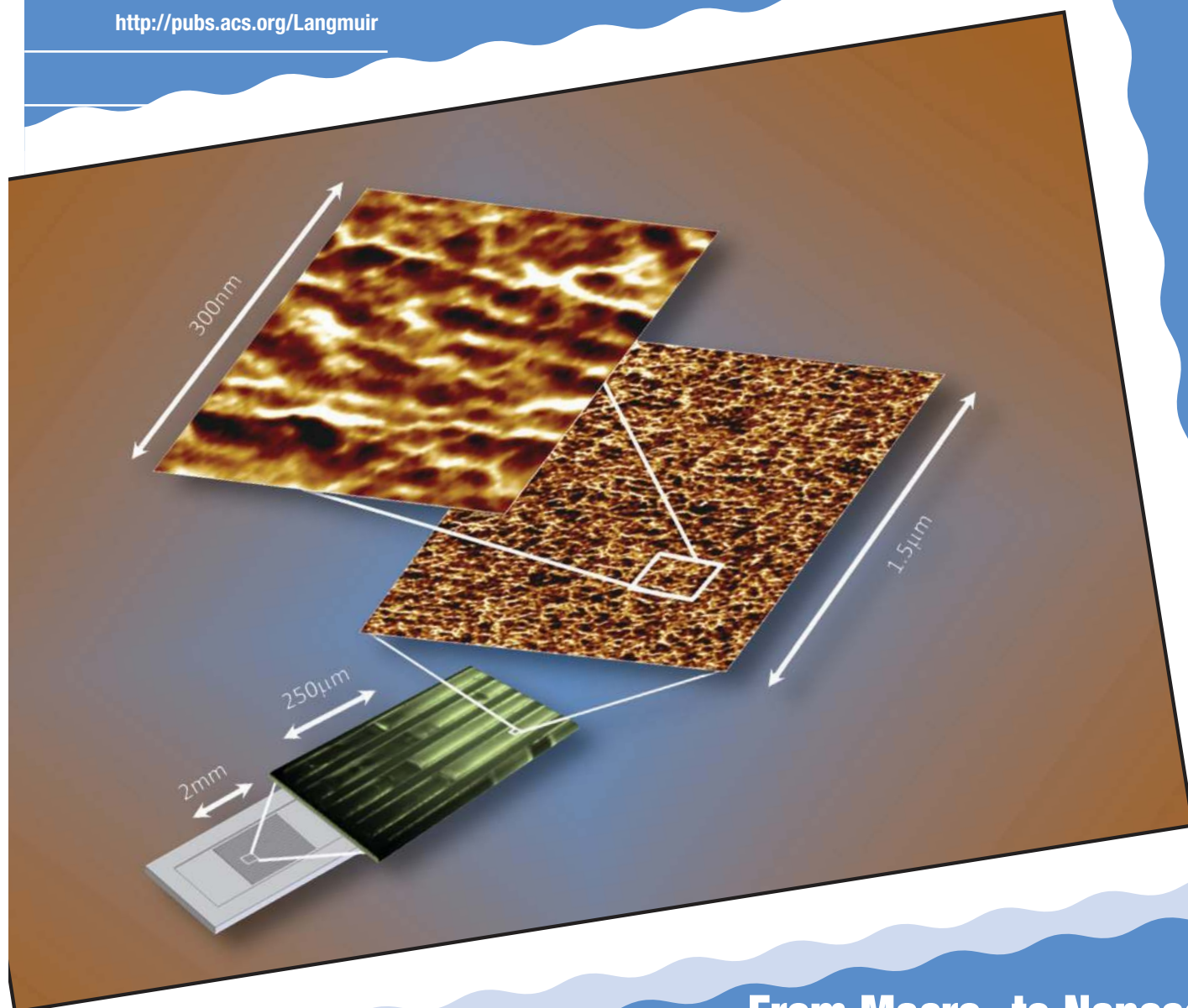
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**From Macro- to Nanoscale
Imaging of Aligned Neurofilaments
Network in Microchannels**

(see p. 28A)

About the Cover: Cover illustration figure by Roy Beck and Holger C. Hesse. Neurofilaments are the major constituents of the neuronal cytoskeleton. In vivo, these filamentous proteins self-assemble to form a dense nematic hydrogel that acts as a scaffold for microtubules and other organelles, and provides structural support and mechanical stability to the neuron. Studying aligned neurofilament networks sheds light on the nature of interactions among neurofilaments as well as on those with the environment. Hesse et al. demonstrate that neurofilaments, while confined in microchannels, self-assemble to an aligned nematic network with an extensive long-range interaction. Using both cross-polarized and atomic force microscopy, they show that neurofilaments align perpendicular to the channel walls, in contrast to other cytoskeleton filaments. This microfluidic technique has the benefit of allowing the properties of the confined filament network to be studied as the local environment is altered. For more information, see “Direct Imaging of Aligned Neurofilament Networks Assembled Using In Situ Dialysis in Microchannels” by H. C. Hesse, R. Beck, C. Ding, J. B. Jones, J. Deek, N. C. MacDonald, Y. Li, and C. R. Safinya on pages 8397-8401 of this issue.

Letters

Direct Imaging of Aligned Neurofilament Networks Assembled Using In Situ Dialysis in Microchannels

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We report a technique to produce aligned neurofilament networks for direct imaging and diffraction studies using in situ dialysis in a microfluidic device. The alignment is achieved by assembling neurofilaments from protein subunits confined within microchannels. Resulting network structure was probed by polarized optical microscopy and atomic force microscopy, which confirmed a high degree of protein alignment inside the microchannels. This technique can be expanded to facilitate structural studies of a wide range of filamentous proteins and their hierarchical assemblies under varying assembly conditions.

Introduction

Network supramolecular assemblies of filamentous proteins are ubiquitous in subcellular structure and are involved in a wide range of biological functions.^{1,2} There is currently an intense effort, both experimentally and theoretically, to study the in vitro reconstitution of filamentous protein networks in order to gain insight into the structural and mechanistic properties and relate this to the in vivo assemblies. The generally high length-to-diameter aspect ratio and rigidity, with a persistence length of over 100 nm, make these biopolymers highly susceptible to alignment by geometric confinement. Achieving oriented samples

is advantageous for both biophysical and structural studies.^{3,4} Here we present a new method for protein alignment, combining in situ microfluidic control of the polymerization of filaments from protein monomers with microchannel confinement. This technique provides flexibility in controlling assembly conditions and requires only minimal amounts of protein. In addition, the alignment allows further structural studies on interfilament interactions.

The microchannel devices were used to investigate the network structure of neurofilaments (NF's), which, together with Actin and microtubules, form the scaffold for nerve cells.¹ Although structural details and functions of NFs and their networks are still poorly understood, it is well-known that NFs consist of three different subunits that self-assemble into a flexible 10-nm-thick filament with unstructured polyampholyte brushes protruding perpendicularly out of the filamentous core.⁵ NFs are an excellent

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example of a filamentous protein with unstructured domains that prevent crystallization for high-resolution structural studies yet form well-ordered networks *in vivo*. The monomer subunits are synthesized in the neuronal cell perikarya and transported, either as subunits⁶ or filament,⁷ into the confinement of neuronal processes (axon and dendrite) with a diameter on the order of micrometers, where they form highly aligned network structures. In several neurodegenerative diseases such as amyotrophic lateral sclerosis (ALS), Lewy-body-type dementia, and Parkinson disease, a disruption of the NF network structure as followed by protein aggregation can be observed.⁸

Previous studies demonstrate that a microchannel device can be used for the alignment of filamentous macromolecules.^{3,4} Here, we expand the microchannel device to create a microfluidic setup that facilitates NF assembly from monomer subunits *in situ*. Highly aligned networks, comparable to those found *in vivo*, assemble from minimal amounts of protein ($\sim 15 \mu\text{g}$) confined inside the channel. This technique has the benefit of allowing the impact of environmental conditions on the properties of the network to be studied without altering filament confinement. Polarized optical microscopy and atomic force microscope (AFM) imaging reveal an aligned NF network inside the microchannels. The orientation is long-range, extending over millimeters, and is surprisingly homeotropic in nature, aligning perpendicular to the channel wall.

Materials and Methods

Microchannel Fabrication. Devices were fabricated on single-side-polished silicon wafers (4 in. diameter, (100) orientation) using standard microfabrication techniques consisting of photolithography and deep silicon (inductively coupled plasma) reactive ion etching.⁹ The patterned wafer was diced into separate rows each holding 11 channel compartments with a size of $2 \times 2 \text{ mm}^2$, which are connected to two loading pools (one on each side of the channel, see Figure 1a). Freshly etched wafers were immersed in piranha solution (50:50 vol% $\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$) for 10 min for cleaning, rinsed with purified water (MilliQ, 82 M Ω , Millipore), and blown dry with nitrogen gas immediately before filling.

Sample Preparation. NF protein was purified from bovine spinal cord according to the method described elsewhere.^{10,11} NF assembly and sample preparation were previously described¹¹ with the following modifications. To form a hydrogel network with high protein concentrations, protein was pelleted by ultracentrifugation at 100 000g for 2 h. This pellet shows the properties of a nematic hydrogel with multiple independent domain directors¹⁰ (Figure 2c). The hydrogel was then separated from the supernatant. We achieved the best results at final protein concentrations of 30 mg/mL ($\sim 3 \text{ wt}\%$ protein) and above.

To introduce the NFs into the microchannels, we add urea salt to the NF pellet to a final concentration of 6–8 M urea, which breaks the network structure and disassembles the filaments into their subunit proteins. After this treatment, about 15 μg of subunit protein in a $\sim 30 \text{ mg/mL}$ stock solution is pipetted into the loading pools, and capillary force draws the solution evenly into the channels. The device is immediately covered with a dialysis sheet (Spectrum Labs, MOCW = 12 000–14 000 Da), and a specially designed dialysis chamber is tightly mounted onto the assembly (Figure 1b,c). The microfluidic device is connected to a peristaltic pump (BioRad, pump speed 1 mL/min), and urea is replaced by a physiological buffer (0.1 M MES pH 6.8, 116 mM NaCl, 6 mM MgCl_2) for 36 h, allowing for complete reassembly of the filaments.

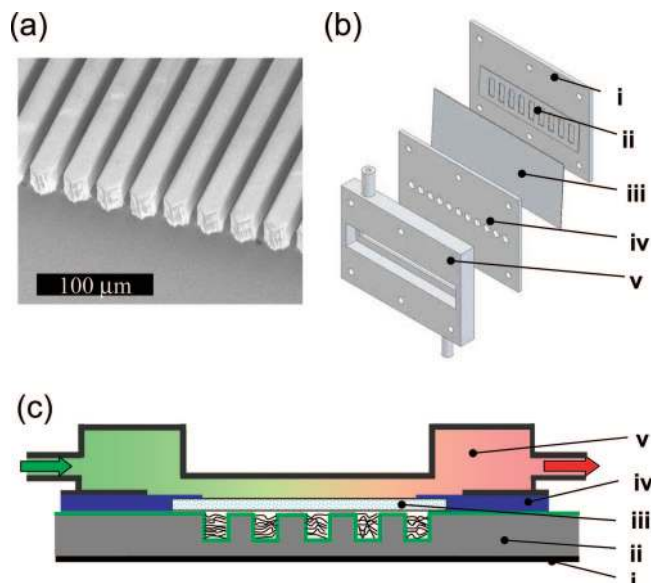


Figure 1. Microchannel in situ dialysis chamber device. (a) Scanning electron micrograph of a 10- μm -wide, 50- μm -deep microchannel compartment opening out into one loading pool. Schematic (b) and cross sectional (c) view of the setup for alignment. Top to bottom: (i) supporting stainless steel plate, (ii) Si wafer with microchannels, (iii) dialysis sheet, (iv) front plate, (v) microfluidic buffer exchange chamber.

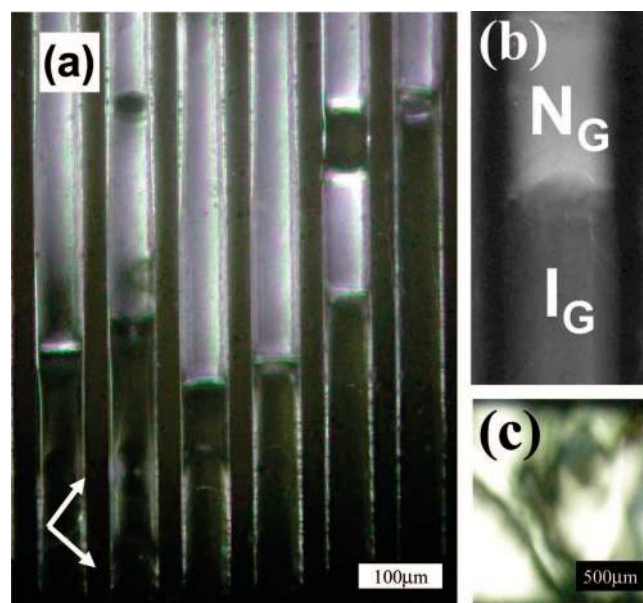


Figure 2. (a, b) Cross-polarized optical microscopy images of an NF hydrogel formed in $50 \times 50 \mu\text{m}^2$ channels. Images were taken at 45° with respect to the analyzer showing bright spots where a nematic hydrogel has formed. (c) NF nematic hydrogel inside a quartz capillary sample.

For AFM imaging, the samples were dried for at least 48 h, washed with purified water, blown dry, and subsequently stored for 2 h in an evacuated desiccator.

Microscopy. Optical microscopy images were taken with a Nikon Coolpix P5000 digital camera mounted on a Nikon Optiphot2-POL microscope equipped with crossed polarizers and a 100 W Hg lamp in reflection mode. The images were analyzed using standard image analysis software.

AFM measurements were conducted using a Digital Instruments Nanoscope IIIa Dimension 3100 and Asylum MFP-3D in ambient tapping mode at a speed of 1 Hz/line. We used AppNano ACL-50 silicon nitride cantilevers with spring constants of 20–95 N/m (f₀

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= 190 kHz) and Olympus AC160TS aluminum cantilevers with a spring constant of 42 N/m ($f_0 = 300$ kHz).

Results

Filament polymerization and network cross-linkage of Actin or microtubules inside microchannels are influenced by interaction with associated proteins and the energy of GTP or ATP hydrolysis.^{3,4} In contrast, the NF system lacks these control parameters. Subunits self-assemble into filaments and, at high concentrations, sidearms of the filaments interact to form a hydrogel with no additional control proteins. With the onset of gelation, the solution becomes too viscous to fill the microchannels.

The presented microfluidic setup uses top-loading dialysis, which allows NF assembly inside the microchannels from their subunits. As urea is replaced by a physiological buffer, subunits polymerize into filaments, and because of the high protein concentrations used, neighboring filaments also start to interact. A highly aligned hydrogel is formed in the confinement of the microchannel walls. The microfluidic device facilitates precise control over buffer conditions, allowing the impact of the environment on the protein hydrogel to be monitored in situ by techniques such as X-ray diffraction.

The NF hydrogel formed in the microchannels was examined by optical microscopy as shown in Figure 2a,b. The birefringent dialysis sheet was replaced by a highly transparent thin film of polydimethyl[siloxane] (PDMS) to prevent sample evaporation. The micrographs show strong birefringence caused by the anisotropy of the rodlike proteins that form a nematic hydrogel. This phase extends across multiple channels with a single orientation vector that exists for a length scale of over a millimeter, indicative of a single domain. In comparison, Figure 2c shows polarized microscopy of a nematic hydrogel inside a 1.5-mm-wide capillary where multiple domain directions and defect structures can be seen. The advantage of the microchannel device for macroscopic uniform alignment can be clearly seen.

Inside the channels in the vicinity of the loading pools, both an isotropic sol (I_S) and an isotropic hydrogel (I_G) is observed, with the latter showing texture in bright-field optical microscopy. Well within the channels at a distance far from the sample loading pools located on the periphery, we find a phase transition into a nematic hydrogel (N_G) characterized by its strong birefringence (Figure 2a,b). This phase transition is likely a function of a protein concentration gradient inside the channels. The device design allows for highly effective dialysis at the center of the channel compartments whereas near the loading pools the dialysis membrane is covered by a PDMS gasket and dialysis is less efficient. Furthermore, the large loading pools cause protein dilution as a result of increased osmotic pressure throughout the dialysis. The different phases shown are in agreement with NF dilution studies based on recent optical and X-ray measurements.¹⁰

Alignment quality and nematic direction can be probed using digital image analysis. The light-scattering intensity of the sample is analyzed at different angles, α , with respect to crossed polarizers. In a uniaxial anisotropic medium, the light intensity, $I(\alpha)$, measured downstream from the analyzer is given by direct application of the law of Malus (derivation in Supporting Information):

$$I(\alpha) = I_0[\sin(2(\alpha - \alpha_0))(1 - \cos(\Delta\phi))]^2 + I_{\text{bck}} \quad (1)$$

Here, I_0 is a multiplication coefficient, I_{bck} is the background intensity, α_0 is the angle between the analyzer and the optical

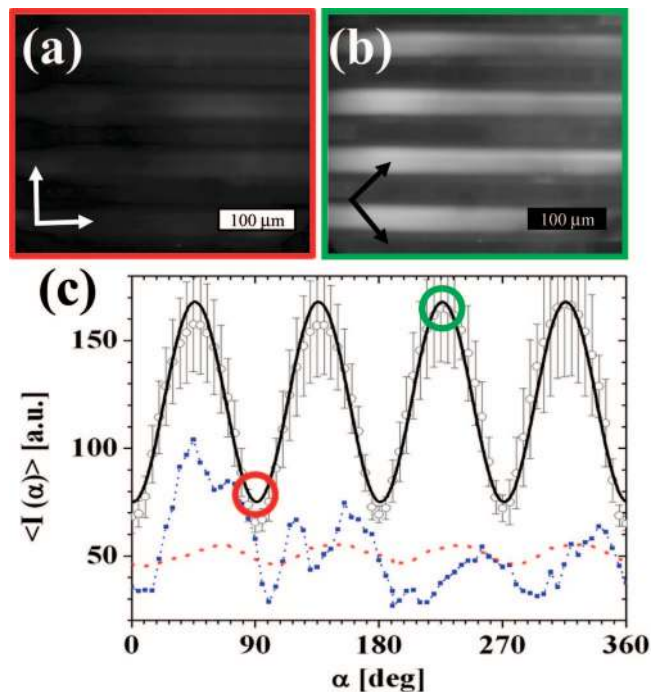


Figure 3. Cross-polarized optical microscopy images of an NF hydrogel formed in $50 \times 50 \mu\text{m}^2$ channels at stage angles of 90° (a) and 225° (b). Arrows represent analyzer and polarizer directions. (c) Cross-polarized light intensity at different stages with respect to polarizer angles. Nematic hydrogel inside a microchannel (black circles) showing strong alignment. The line is a fit to eq 1. Capillary sample (dotted blue line) and isotropic gel in microchannels (broken red line) showing no preferred orientation. Error bars are standard deviations of overall light intensity in each image.

axis of the filaments, and $\Delta\phi$ is the phase shift of the anisotropic media. For aligned rods, $\Delta\phi = 2\pi d\Delta n/\lambda$ where Δn is the retardance between the ordinary and extraordinary rays, which is related to the protein concentration,¹² d is the effective length that light travels through the anisotropic medium, and λ is the light wavelength.

In Figure 3a,b, we show polarized microscopy images at the two intensity extremes. Figure 3c shows the averaged light intensity $\langle I(\alpha) \rangle$ measured on a fixed area of $200 \times 40 \mu\text{m}^2$ inside the channels, avoiding spurious birefringence from the channel wall. The results fit well to eq 1 and prove the alignment of the filaments inside the channel. We find a tilt of $\alpha_0 = 2.0 \pm 0.7^\circ$. Therefore, within our experimental resolution, the alignment is found to be either parallel or perpendicular with respect to the channels walls. In comparison, macroscopic measurements and analysis of NFs in the nematic phase inside a capillary show arbitrary light-intensity fluctuations and a large standard deviation ($\Delta I(\alpha)/I(\alpha) > 1$ for all α) as we average over multiple domains with different nematic director orientations (Figure 3c, dotted line). Inside the loading pools and in the vicinity of the microchannels, the analysis shows only background intensity consistent with the presence of isotropic gel and isotropic sol phases (Figure 3c, dashed line).

This simple optical analysis method allows us to gain insight into macroscopic filament alignment. However, because of the 4-fold symmetry nature of the data, we cannot distinguish between homeotropic and planar orientations. Scanning probe AFM measurements inside the microchannels give higher-resolution images and microscopic details that allow a distinction to be made between the two possible filament orientations.

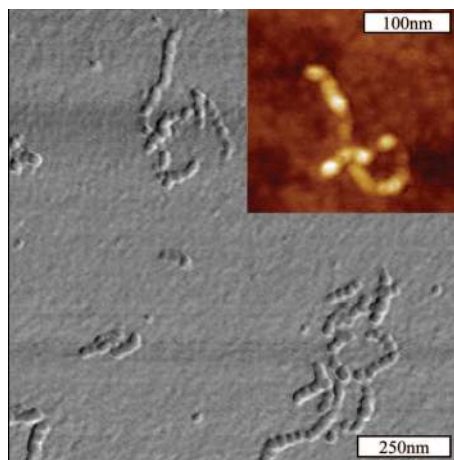


Figure 4. AFM phase image of dilute NFs deposited on a PEI-coated silicon wafer. The inset shows a height image with the same topographic features.

A typical AFM phase image of single NFs deposited on a polyethyleneimine (PEI)-coated¹³ silicon wafer is shown in Figure 4. The micrographs clearly show the characteristic bead structure of the NF backbone with about a 45 nm repeat. The height trace reveals a filament cross section of 3 nm in height and 50 nm in width due to surface effects and tip convolution as reported elsewhere.¹⁴

To reveal the microscopic ordering of the NFs inside the microchannels, we probed several channels using polarized optical microscopy and scanning probe AFM. Figure 5 shows a typical AFM phase image taken inside a 50- μm -wide microchannel. Both phase and height traces of the hydrogels are highly reproducible and show the same typical bead structure and cross section as observed on single filaments. However, because of the relatively large height modulation of the overall network structure, the texture is more readily observed in the phase images. Interestingly, the filament alignment inside the microchannel compartment is found to be homeotropic with respect to the channel axis. The strong perpendicular alignment was confirmed by all measurements on nematic gels inside the microchannels and is in complete agreement with the birefringence analysis. As expected, AFM imaging inside the loading pools does not reveal any filament alignment; control AFM images inside freshly etched and cleaned microchannels show only random defect structures (Supporting Information, Figure S1).

Discussion

With a persistence length shorter than 1 μm , NFs are extremely flexible polymer chains.¹⁴ One would not expect a powerful alignment effect on the sample because of our comparatively loose microchannel confinement at 10 to 100 μm . Interestingly, a very strong orientation effect is evident with microchannels of 50 μm width and 50 μm depth. This phenomenon might be explained by looking at the aggregation process in detail. Planar alignment of the other cytoskeleton filaments, microtubules, and actin by microchannel confinement has previously been reported.^{3,4} This is opposite to the orientation seen with NFs where filaments align perpendicular to the channel wall. Persistence and contour lengths of the NFs

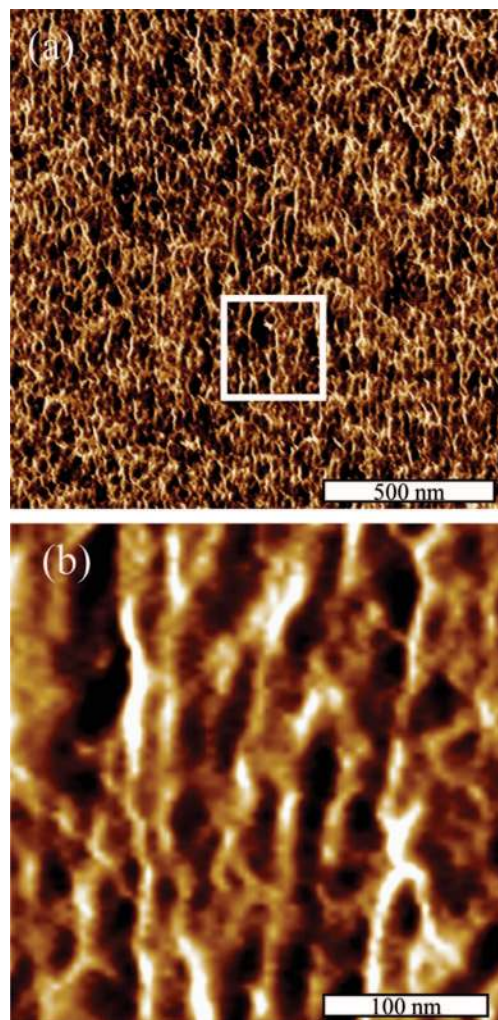


Figure 5. AFM phase images on an aligned nematic hydrogel inside a 50- μm -wide microchannel. The right image close-up is 300 nm^2 . A Fourier transform analysis of image a is shown in Supporting Information, Figure S2.

are at least 1 order of magnitude smaller than for the cytoskeletal filaments explored in other confinement studies. This allows the NFs to rotate freely within the relatively wide channels to their preferred orientation. Moreover, in contrast to microtubules and Actin, the results demonstrate that the relevant length scale responsible for the NF alignment is neither the single filament persistence nor the contour length, suggesting that filament network interactions are causing much longer range orientation that extends to the width of the channels. Recent mechanical studies on neurofilament hydrogels suggest that axial sliding of the building blocks might be possible throughout the gel-formation process.¹⁵ Because of sidearm interaction, bundles of filaments with a contour and persistence length significantly larger than that of a single filament could play a key role when filaments assemble to a gel.

Additional mechanical and environmental factors may contribute to the preferred NF alignment. For instance, the interaction energy between the NFs and Si channel wall may be minimized by perpendicular orientation possibly as a result of electrostatic and steric repulsions of the radiating unstructured brushes to the untreated silicon substrate. This is supported by X-ray observations of nematic NF samples inside quartz capillaries where a

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weak preferential perpendicular orientation with respect to the capillary walls is seen.

The aggregation of protofilaments and the formation of a hydrogel strongly depends on buffer and dialysis conditions. The microfluidic device allows in situ filament assembly under varying, but well-defined, buffer conditions. Thus, using the microfluidic device, the filament length,¹⁶ and phase appearance¹⁰ of the solution can be influenced. X-ray diffraction studies with the microchannel assembly mounted in transmission geometry are currently in progress and will be described in a future communication. In addition, our technique allows for a variety of other new studies. For example, the microchannel design can be used for in situ motility assays on a nematic hydrogel of NF from one pool to the other. Thus, a more quantitative understanding of neurodegenerative diseases such as ALS might become possible. In a typical nerve cell, the NFs tend to align parallel to the axon in a much smaller confinement. Therefore, mechanisms such as phosphorylation and an infrastructure of stiff microtubules might be needed in early stages of axon development.

Conclusions

We have shown that a combination of a microchannel device and microfluidic dialysis allows us to align a hydrogel of filamentous proteins such as NFs. We demonstrated the quality of the macroscopic alignment using polarized microscopy. The details of the microscopic structure and the unexpected per-

pendicular alignment were probed using AFM inside the microchannels. Polymerizing the filaments in the microchannels, as demonstrated here, allows further in-depth investigations of the structure and interactions of NFs, using, for example, X-ray diffraction and motility assays.

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Supporting Information Available: Derivation of eq 1. AFM scans of nonordered neurofilament hydrogel structures and random structures present after etching and cleaning. Fourier transform analysis of Figure 5a. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Supplementary material:

Derivation of equation (1). Following the law of Malus, let us assume an electromagnetic wave with wave vector k , and angular frequency ω propagating in direction of the z axis. After passing the polarizer we assume linear polarized light in the x axis and the electric field will have the form:

$$\vec{E}_{init} = \vec{E}_{x,0} e^{i(kz - \omega t)}$$

An uniaxial anisotropic medium is placed on the rotating microscope stage with its optical axis at angle α_0 with respect to the orientation of the polarizer. We measure the intensity at different stage angles α . The incident electric field wave in the coordinate system of the anisotropic medium is therefore:

$$\vec{E} = \begin{pmatrix} \vec{E}_{\parallel} \\ \vec{E}_{\perp} \end{pmatrix} e^{i(kr - \omega t)} = \vec{E}_{x,0} \begin{pmatrix} \cos \alpha^* \\ \sin \alpha^* \end{pmatrix} e^{i(kr - \omega t)}$$

With $\alpha^* = \alpha_0 + \alpha$ and E_{\parallel} and E_{\perp} denote the field parallel and perpendicular to the optical axis. While traveling through the anisotropic medium a phase of $\Delta\phi = 2\pi d\Delta n / \lambda$ [S1] is accumulated. Therefore the electric field after the anisotropic media is:

$$\vec{E}' = \vec{E}_{x,0} \begin{pmatrix} \cos \alpha^* \\ \sin \alpha^* e^{i\Delta\phi} \end{pmatrix} e^{i(kr - \omega t)}$$

Transformation back to our original coordinate system gives:

$$\begin{aligned} \vec{E}_x &= \vec{E}_{x,0} (\cos^2 \alpha^* + \sin^2 \alpha^* e^{i\Delta\phi}) e^{i(kr - \omega t)} \\ \vec{E}_y &= \vec{E}_{x,0} (\sin \alpha^* \cos \alpha^* - \cos \alpha^* \sin \alpha^* e^{i\Delta\phi}) e^{i(kr - \omega t)} \end{aligned}$$

After the Analyzer, oriented 90 degree to the polarizer in the y -plane, the light intensity is therefore

$$I(\alpha) = |\vec{E}_y|^2 = |\vec{E}_{x,0}|^2 [\sin(2(\alpha - \alpha_0))(1 - \cos(\Delta\phi))]^2$$

Reference:

S1. Gerthsen, C. in *Gerthsen Physik*; Springer-Verlag 22. Edition, **2004**

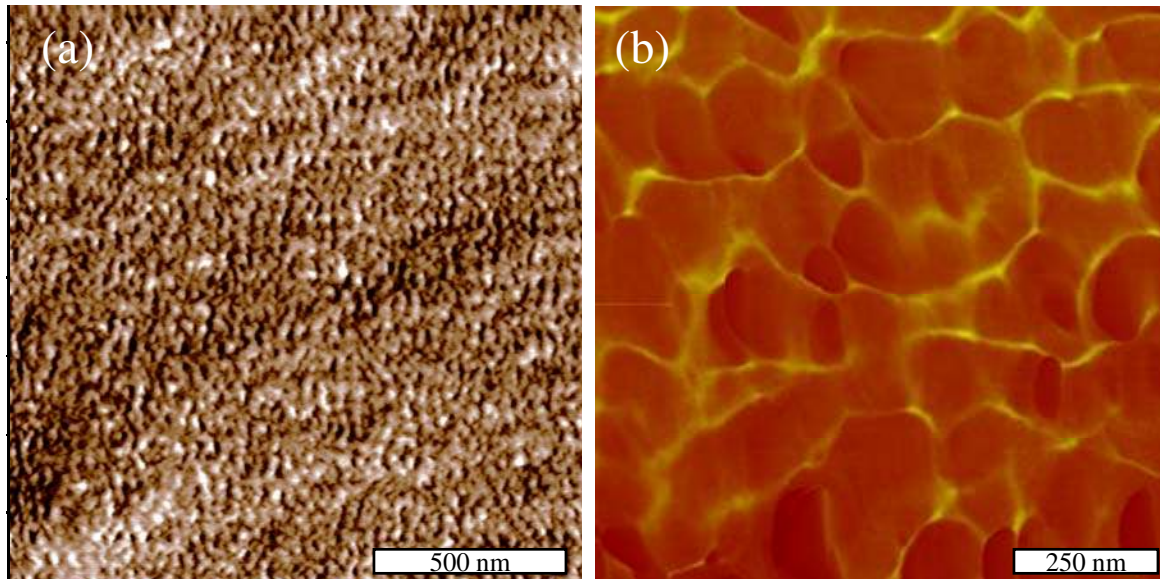


Fig. S1: (a) AFM scan of non ordered neurofilament hydrogel structures imaged in the outermost parts of a microchannel. Optical microscopy at this spot confirms a isotropic gel phase with no birefringence. (b) Random structures present both inside the pools and at the trenches of the channels after reactive ion etching and subsequent piranha cleaning.

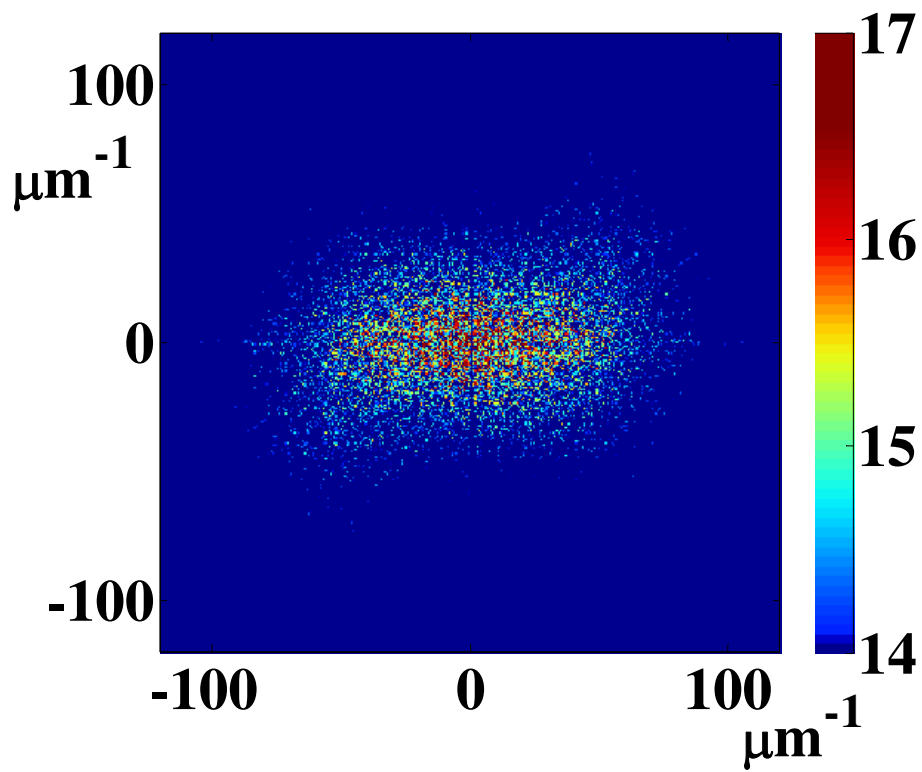


Fig. S2: Fourier transform analysis of image 5(a) showing a preferred orientation perpendicular to the channel walls and an inter-filament separation in the order of a few tens of nanometers.