

Microchannel Systems in Titanium and Silicon for Structural and Mechanical Studies of Aligned Protein Self-Assemblies

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We report a technique for the alignment of self-assembled protein systems, such as F-actin bundles and microtubules, in a surface-modified titanium or silicon microfluidic device. Assembling filamentous protein systems in a confined geometry produces highly aligned samples for structural and mechanical studies. Biomolecular self-assembly can be investigated in a controlled fashion under different molecular concentration gradients and conditions along a channel length. We have shown that surface-modified devices produced via a high aspect ratio etch process in titanium and silicon can be used to confine and control such macromolecular assemblies and present examples of F-actin bundles and microtubules in this system.

Introduction

The study of self-assembled protein systems and how their structure relates to function is part of the rapidly expanding field of proteomics and will require new techniques for the confinement and manipulation of specific molecular assemblies. In this paper we discuss a device which will facilitate the study of filamentous proteins and associated molecules and show how we have introduced such self-assemblies into a confined geometry.

The eukaryotic cell cytoskeleton is composed of a complex network of actin, microtubules and intermediate filaments. These filamentous proteins self-assemble to form many different structures in the cell. For example, actin filaments will form bundles or sheetlike cross-linked structures on interacting with actin binding proteins (ABPs). Microtubules may be cross-linked via microtubule-associated proteins (MAPs) or bind with the protein kinesin to facilitate vesicle transport in the cell.¹ These examples of multi-protein structures occur under specific environmental conditions, and a delicate balance of localized chemical concentrations can control their assembly or disassembly. If we are to aim to study protein self-assemblies, then great care must be taken to observe them under physiological conditions. Relatively destructive techniques such as transmission electron microscopy (TEM) allow us to observe structures in a dried state or after chemical treatment; however geometrical analysis of such images may be misleading.

Biological self-assembled systems can be very delicate structures, and formed under physiological conditions *in vitro*, they are extremely difficult to manipulate. The F-actin/ α -actinin bundle system is one such example. These loosely packed bundles of F-actin filaments cross-linked by the α -actinin molecule have an inter-fiber

spacing of ~ 35 nm. These bundles will branch in solution to fill space as a three-dimensional network of bundles.² The most effective method to study delicate multi-protein systems such as this may be via X-ray scattering from dilute solutions. In this way, solution concentrations can be carefully controlled to give an accurate observation of structural ordering under physiological conditions. The main problem with this technique is that self-assembled protein structures tend to be composed of large molecules and weakly ordered. This means any X-ray scattering signal will be extremely difficult to detect without using concentrated samples. Many protein samples are costly and difficult to purify, thus limiting the concentrations of protein available for study.

In this paper we describe a technique by which it is possible to investigate the structural and mechanical properties of self-assembled protein systems by confinement in a microchannel system. This technique allows self-assembly to occur under defined conditions while minimizing external influences, e.g., shear forces or attraction to a surface. By forming actin bundles (or other filamentous structures) in a narrow channel, where the width of the channel is smaller than the persistence length of the structure, we have produced highly aligned protein self-assemblies. The alignment of self-assembled systems is a difficult problem to overcome due to the inherent fragility of such materials. Structures such as cytoskeletal bundles or DNA complexes will form only under specific conditions and cannot be crystallized for detailed structural studies. Most of the X-ray scattering work on such systems to date has been with unoriented (powder) samples. While such work is able to provide some information on molecular spacings in a complex, much more detailed work is possible with aligned samples as structures with 2D ordering can be clearly resolved.

We aim to produce aligned biomolecular self-assemblies at relatively low concentrations. The alignment will not only provide extra information on the molecular structure

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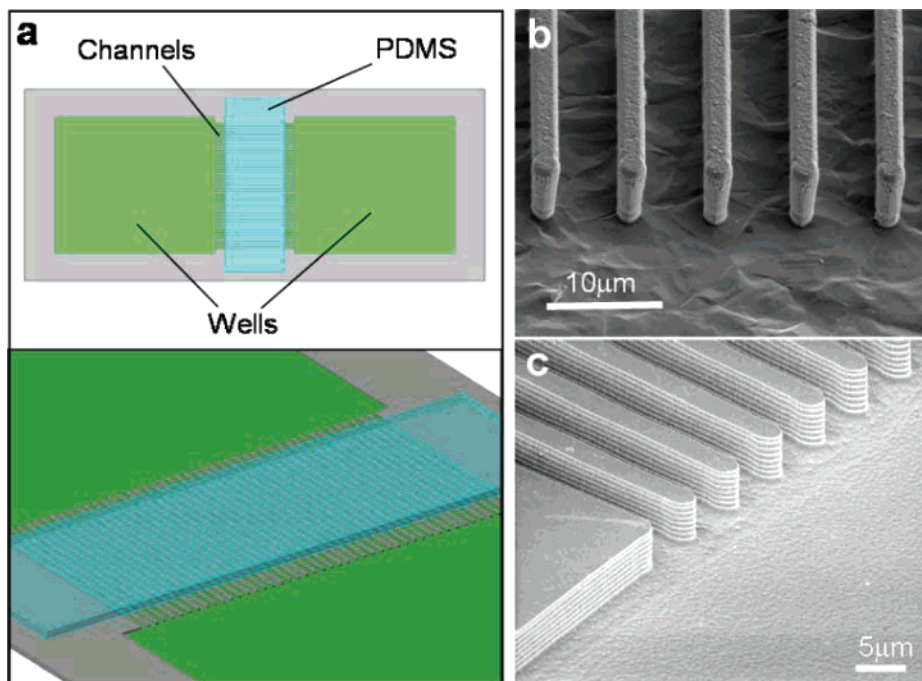


Figure 1. (a) A schematic of the microchannel device and SEM images of the channel ends in (b) titanium and (c) silicon.

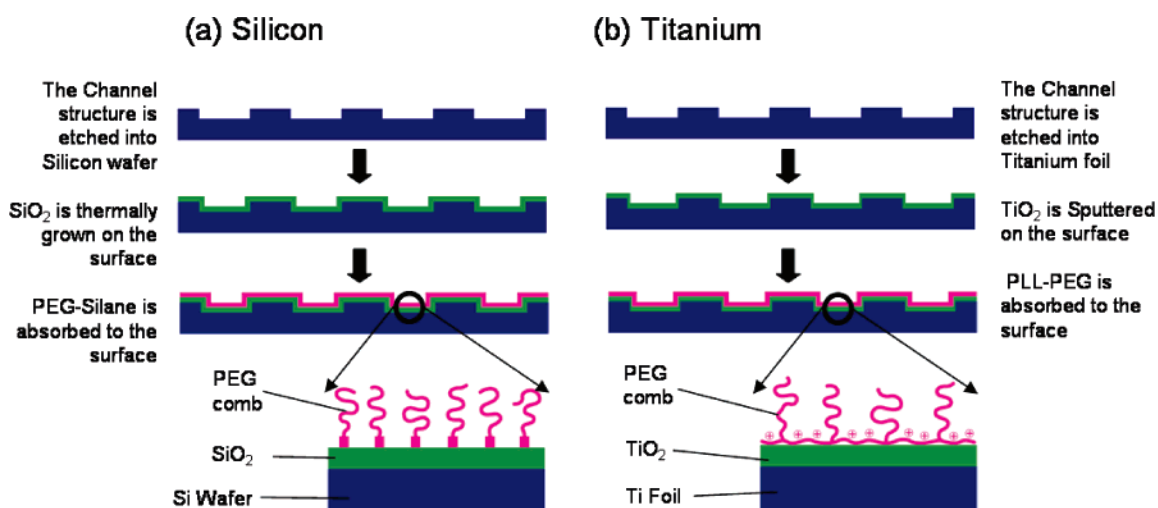


Figure 2. Schematics for the preparation of (a) silicon and (b) titanium microchannels.

of an assembly, but will also enhance our signal-to-noise ratio allowing lower concentration solutions to be investigated. By aligning assemblies in a confined environment, we are able to study their mechanical and structural properties in detail. Typical X-ray scattering samples in such systems consist of a concentrated pellet of material, produced by centrifugation. The production of such a sample often requires a large volume of protein to produce. We aim to carry out X-ray scattering studies on very small volumes of material. This technique has been demonstrated on a silicon device,³ to give a highly aligned X-ray diffraction pattern for the α -actinin/actin bundle system.

Such devices will be useful for X-ray structural investigations and also as a system which allows the easy investigation of the mechanical properties of self-assembled systems in a controlled environment. We have seen previously the interesting structures formed by branching actin bundles on the microscale.⁴ By confining

material in a controlled way, we are able to more clearly observe and study protein microstructures. Titanium is an interesting material to work with for microdevice fabrication. The metal is biocompatible and already utilized widely in medical implants. The titanium deep etch process is relatively new and provides us with a material which is extremely robust. Devices can be thinned to as little as $5\ \mu\text{m}$ while retaining their structural integrity and patterned with small features comparable to those commonly produced in silicon. A system designed to carry out force measurements can be incorporated into the microdevice,^{5,6} and titanium lithography provides an ideal substrate for such systems as the metal is flexible and conductive. The surface of our device can be modified to resist protein absorption, and we describe methods by which this can be achieved for both titanium and silicon systems.

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Materials and Methods

Microchannel Design and Fabrication. The microdevice was designed to have two large reservoirs, connected by an array of narrow channels. These channels were varied in width and length. For our experiments we used channels 5, 10, and 20 μm wide, 10 μm deep, and 800 or 1600 μm long (Figure 1). The devices were etched into polycrystalline titanium foil or silicon wafers, and scanning electron microscopy (SEM) images of the channels opening out onto a reservoir area can be seen in Figure 1b,c for both titanium and silicon, respectively. The reservoir depth can also be varied independently of the channel depth, although the SEM images show single-depth devices only.

Recent developments have allowed for the etching of high aspect ratio structures into bulk titanium.⁷ Titanium channels were prepared using 99.6% annealed titanium thin foils⁸ approximately 25 μm thick. Each foil was chemically mechanically polished and then soaked for 8–12 h in organic soap and cleaned using acetone and 2-propanol with ultrasonic agitation. Following cleaning, a TiO_2 etch mask was deposited on the foil using reactive sputtering. The titanium was deep etched via the TIDE process,⁹ resulting in a smooth, deep etch. Following the deep etch, an additional 0.4 μm TiO_2 film was sputtered onto the channels to improve hydrophilicity. The backside of the titanium foil was deep etched to further reduce the substrate thickness and therefore reduce X-ray attenuation. Average substrate final thicknesses were found to be less than 5 μm under the floors of the channels and wells. Following the backside etch, the foils were once again cleaned using an O_2 plasma. An example of the titanium channel structure can be seen in Figure 1b.

The silicon microchannels were fabricated using a similar high aspect ratio etch process.¹⁰ The deep channel structure in Si is fabricated using inductively coupled plasma (ICP) etch cycles with SF_6 and C_4F_8 gases through an SiO_2 mask. The etch cycles result in a ridged structure on the channel walls, as can be seen in Figure 1c. Devices in titanium however have much smoother sidewalls, a feature which is somewhat advantageous for X-ray applications and experiments using large molecules. The floor of the silicon channels is smoother and more uniform than that of the titanium; however we do not believe that floor roughness on this length-scale significantly impacts the protein systems studied in this paper.

Surface Modification. To allow easy filling of the channel system with aqueous protein solutions, the channel surfaces must be hydrophilic. To allow protein self-assembly to occur within the channel while minimizing interactions with the surfaces, the channel walls were coated with a brush¹¹ of poly(ethylene glycol) (PEG), a material well known to resist protein absorption.

In titanium devices these properties were achieved by the deposition of a sputtered film of TiO_2 onto which poly(L-lysine)-*g*-poly(ethylene glycol) (PLL-*g*-PEG) was deposited. PLL-*g*-PEG is a polycationic PEG graft copolymer which has been shown to chemically absorb onto anionic surfaces. This molecule has been shown to provide a high degree of resistance to protein absorption on metal oxide surfaces, including titanium oxide.¹² The surface of the titanium channels is strongly anionic; therefore in order to reduce the interaction of proteins with the surfaces a layer of PLL-*g*-PEG was absorbed to the surface. The poly(L-lysine) backbone of the molecule interacts electrostatically with the negatively charged substrate creating a comblike structure of PEG molecules on the titanium oxide surface. The graft copolymer was synthesized from 20 kDa poly-L-lysine and 2 kDa PEG as the protein absorption suppression has been found to depend on

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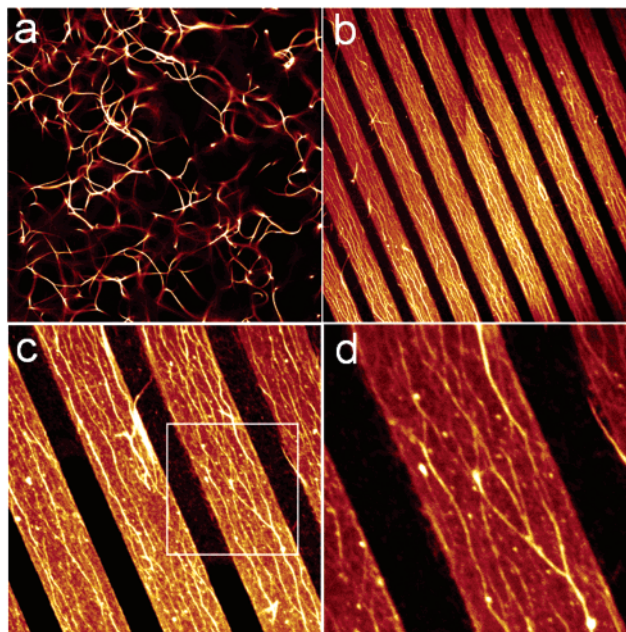


Figure 3. LSCM images of F-actin bundles formed in the presence of α -actinin at a molar ratio of 1:5 α -actinin/G-actin (a) with no confinement and (b–d) in 10 mm silicon microchannels. Panel d shows a close-up as marked in panel c.

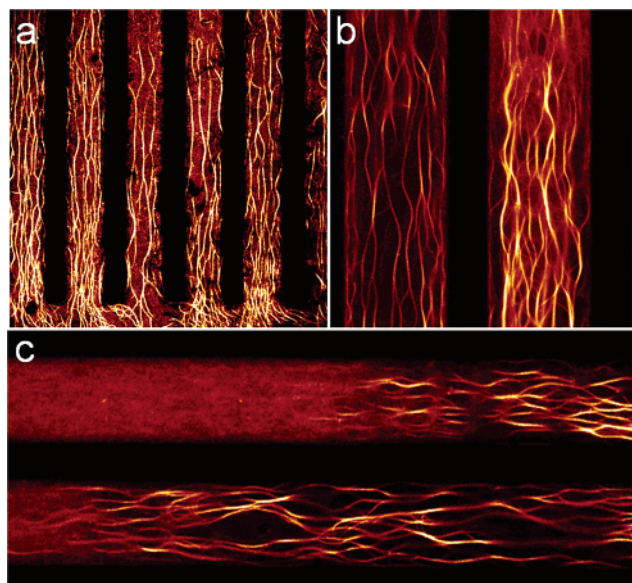


Figure 4. F-actin bundles formed in the presence of α -actinin in 20 μm titanium microchannels.

the molecular weight of the PEG and poly(L-lysine).¹³ Silicon channels were modified by thermal oxidation of the surface followed by the deposition of PEG-silane molecules.¹⁴ This molecule has a similar effect to that described above for titanium, functionalizing the silicon surface with PEG molecules. A summary of the surface modification process in both materials can be seen in Figure 2. The channel array was covered with a 20 μm poly(dimethylsiloxane) PDMS film. This film helped to prevent evaporation from the device and enabled easy viewing of samples under the microscope.

Protein Preparation. Two different protein systems were investigated in this work, α -actinin/actin bundles and micro-

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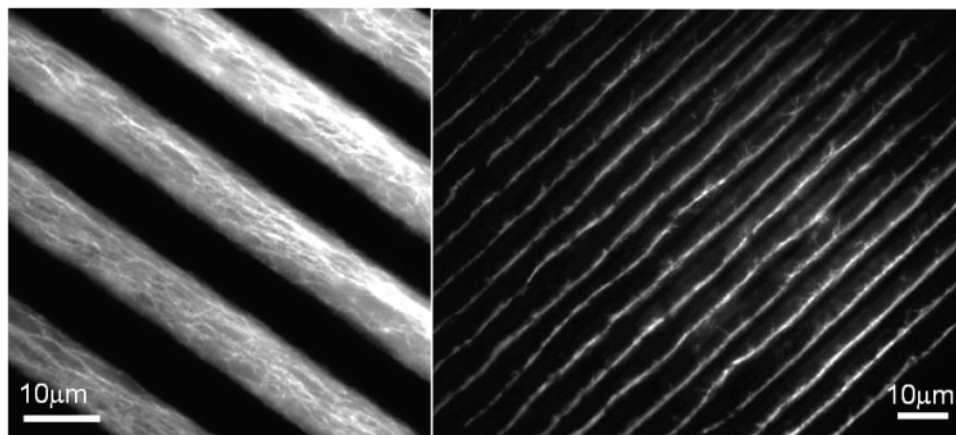


Figure 5. Bundles assembled from prepolymerized 300 nm F-actin filaments with α -actinin in (a) 10 μm wide and (b) 5 μm wide silicon microchannels.

tubules. G-Actin was obtained from Cytoskeleton Inc. and diluted with G-buffer (5 mM Tris-HCl pH 8.0, 0.2 mM CaCl_2 , 0.2 mM ATP, and 0.5 mM DTT) to the required concentration. Polymerization to F-actin was achieved by the addition of KCl to 100 mM final concentration. The actin binding protein, α -actinin (Cytoskeleton), was suspended in 50 mM NaCl, 20 mM PIPES pH 7.2 and 15 mM β -mercaptoethanol.

Microtubules were prepared in PEM buffer (100 μm PIPES, 1 mM EGTA, and 1 mM MgSO_4), by combining fluorescent tubulin (Cytoskeleton) with 5% glycerol and 100 μm GTP, and the microtubules were stabilized by the addition of a 1:1 ratio of taxol to tubulin.

Microscopy. The laser scanning confocal microscopy (LSCM) imaging was carried out on a Leica TCS MP confocal microscope in reflection fluorescence mode. Fluorescent G-actin¹⁵ was excited via an Ar ion laser (488 nm). Other fluorescence images were taken via an inverted fluorescence microscope with a xenon lamp illuminating the sample at 568 or 488 nm and a Hammamatsu Sencicam high-resolution digital camera.

F-Actin Bundles

To obtain highly aligned actin bundles in the microchannels, two different filling methods were investigated. The first method involved in situ polymerization of G-actin in the presence of the linker protein, α -actinin. This technique most accurately represents the process of bundle formation in vivo. G-Actin was first added to one of the side wells (see Figure 1), and the channels filled with actin solution via capillary flow. An α -actinin solution was then added to the opposite well, diffusing into the G-actin along the length of the channel. α -Actinin will not bind with G-actin alone, but only with the polymerized form, F-actin. In order for bundles to form therefore, KCl solution was added to the well containing the G-actin solution. G-Actin will polymerize into the filamentous F-actin form at a KCl concentration of 100 mM. The concentrations of actin and α -actinin solutions can be varied. In this paper we used an average molar ratio of α -actinin to G-actin of 1:5.

After 30 min the channels were examined via confocal microscopy and highly aligned bundles were observed in the microchannels in both titanium and silicon devices. Figure 3b–d shows confocal images of fluorescent actin bundles polymerized in a silicon microchannel device. It is clear, on comparison with the image of bundles formed without confinement (Figure 3a), that the use of a channel system has resulted in alignment of the protein structure. The alignment cannot be a flow effect as we fill the channels with the unpolymerized G-actin molecules initially. Instead alignment must be induced by either a

concentration gradient or more likely geometric confinement of fluctuations as the bundles form.

Figure 4 shows examples of 20 μm wide, 10 μm deep titanium channels filled with α -actinin/actin bundles. This figure shows the bundles in the channel very clearly. Thick individual bundles can clearly be resolved, and in Figure 4b we can see curvature in the bundle structure as it lies in the channel. Many branch points can also be observed. It is interesting to note that the bundles appear to have formed in response to a gradient, as highlighted by Figure 4c. Thick bundles have formed at the channel end close to the α -actinin reservoir, apparently reducing in thickness toward the actin reservoir. This indicates that bundle thickness is a function of α -actinin/actin ratio in solution, a result observed in bulk samples also,⁴ and that the rate of diffusion for α -actinin in an actin solution plays a role here.

An alternative filling method was also investigated, in which actin filaments were prepolymerized to an average length of 300 nm. The channel was then filled with this F-actin solution, and α -actinin solution was introduced to the opposite well in the device. This method worked well also, although it was found that the degree of alignment was greater for the in situ polymerization method and the channels were less likely to clog with material on filling, a problem observed with prepolymerized actin (Figure 5).

By confining self-assembled structures in a microchannel array, it can be clearly seen from the images in Figure 4 that this technique will be extremely useful for mechanical and kinetic studies of the assemblies, in addition to the X-ray scattering studies discussed earlier. We are able to image and manipulate large biomolecular structures, controlling their solution concentrations and behavior under molecular gradients.

Microtubules

Another interesting protein system to study via alignment is that of microtubules and their associated proteins (MAPs). As with the linker proteins which may attach to the actin filament, it is also possible to study the arrangement of MAPs along the microtubule, if we can align this system for X-ray scattering in the same way. Microtubules were polymerized in titanium microchannels, and the results can be seen in Figure 6. A solution of 4 mg/mL tubulin in a suitable buffer for polymerization was introduced to channels of different thicknesses on ice and then incubated at 37 $^\circ\text{C}$ to induce polymerization. It was found, as can be seen in Figure 6, that the microtubules polymerized into an aligned state in 5 μm

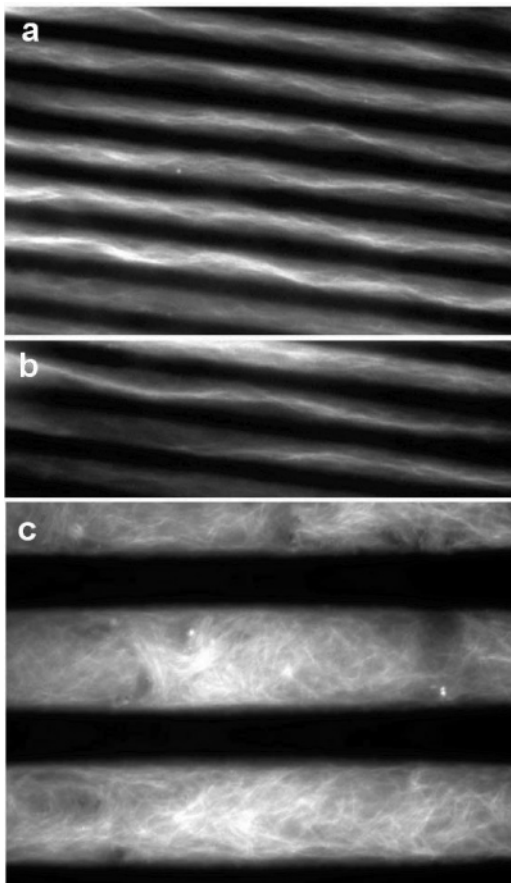


Figure 6. Fluorescence microscopy images of microtubules polymerized in situ in 5 μm wide (a,b) and 20 μm wide (c) titanium channels.

wide channels; however in 20 μm channels very little alignment was observed. Microtubules are fairly stiff, with a persistence length of $\sim 20 \mu\text{m}$; therefore we expect to see alignment as we move to a channel width smaller than the persistence length. This effect was evident as channel width was reduced in our experiments.

Conclusions

We have designed a system based on microfabrication of titanium and silicon channel arrays for the confinement and alignment of filamentous self-assembled protein structures. The channel surfaces in both materials have been modified with PEG molecules to resist protein absorption. F-Actin bundled with α -actinin and microtubules have been assembled in both titanium and silicon microchannels, and a high degree of alignment was observed. Such macromolecular self-assembled structures cannot be easily manipulated, and we have developed a technique by which they may be controlled for further study, be it X-ray scattering, force measurements, or application of electric fields.

For potential X-ray scattering applications, we have found that the polycrystalline titanium substrates show significant advantages over silicon wafers in both background scattering and robustness. Titanium is also a good thermal and electrical conductor and can be patterned to give suitable surface and dielectric properties.

This channel system shows excellent potential for the study of low concentrations of protein self-assemblies. Using shallow channels, one can look at the molecular dynamics of actin bundles and single filaments or microtubules and other filamentous proteins in a controlled way via fluorescence microscopy. Chemical gradients can be applied along the channels and electric field conditions controlled by micropatterning the device with insulating and conducting surfaces.

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