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Protein-based nanotechnology: Kinesin-microtubule driven systems for bioanalytical applications

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I. Introduction

Protein machines carry out tasks critical to cell function, including DNA replication, intracellular transport, ion pumping, and cell motility. They have evolved incredible diversity, specificity, efficiency, and precision, and a considerable proportion of research in modern biology aims to uncover the fundamental mechanisms underlying their function.[1] The cytoskeletal motors, kinesins, dyneins, and myosins constitute a subset of these protein machines, and are notable in being able to convert chemical energy directly to mechanical work. In cells, these motors generate the force that drives muscle contraction, they transport intracellular cargo throughout cells, and they drive the critical movements that underlie cell division.

With the ability to engineer devices and systems at the micron and submicron scales and to synthesize nanoparticles with novel and powerful functionalities, there is a growing need to transport and organize material at submicron dimensions. Because cytoskeletal motors have evolved specifically to transport and organize material at these size scales, there is a current effort to integrate these molecular motors and their cytoskeletal tracks into engineered devices.[2-10] This interdisciplinary research, which involves biologists, bioengineers, chemists, materials scientists, electrical engineers, and others is part of a larger effort to integrate proteins with highly evolved functions into nanoscale engineered systems. For instance, proteins and peptides are also being used as tools to drive self-assembly of inorganic materials such as semiconductors into functional materials.[11,12] Another example of protein based nanotechnology is the push to create electronic devices based on proteins or ion channels.[13]

Compared to pressure-driven microfluidic flow, active transport by molecular motors in microfluidic channels offers a number of advantages as components of bioanalytical systems or biosensors. First, because of the small size of the motors and cytoskeletal filaments, the channels can be scaled down to dimensions below 100 nm. Second, motor-driven transport can occur up concentration gradients and against fluid flows. Third, because energy for transport (in the form of ATP) is delivered and consumed directly at the site of transport, dense arrays of multiplexed channels can be created without need for complex electrical connections. However, before biomolecular motors can be integrated into hybrid micro and nanoscale engineered systems, there are a number of experimental hurdles that must be tackled. First, interfaces between the proteins and material surfaces must be optimized to attach proteins while maintaining their

biochemical function. Second, the design of these microsystems needs to be optimized to best capitalize on the unique transport properties of these motors. Finally, to make analytical devices based on biomotors a reality, it is crucial to develop methods for attaching designated cargo to these proteins and to increase the stability of these proteins.

This chapter focuses on the integration of kinesin molecular motors and their microtubule tracks into microdevices for bioanalytical applications. A number of insightful reviews have been written on the molecular mechanism of motor proteins in general,[14,15] and kinesin motors in particular.[16,17] There are also reviews on applications of biomolecular motors in nanotechnology[18,19] and on applications of kinesin motors in microscale transport.[4] Finally, there is a parallel effort underway using actin and myosin for transport in microscale and nanoscale transport applications,[20-23] but that work is not discussed here.

This chapter is organized as follows. In Section II, the relevant cell biology and biophysics of the kinesin-microtubule system is presented, including a description of the *in vitro* assays that have been developed to study kinesin function. Section III explores theoretical aspects of motor-driven microscale transport, in particular the relationship between transport speeds and diffusion times for particles of various sizes. This analysis helps to frame applications in which kinesin-driven transport is best utilized. Section IV discusses experimental approaches to interfacing motor proteins and microtubules with engineered surfaces. Section V reviews approaches that have been taken to control the direction of kinesin and microtubule movements. These studies provide the core work that needs to be accomplished towards integrating motor proteins into functional microscale devices. To create bioanalytical systems or biosensors driven by the kinesin-microtubule system, it is also crucial to develop strategies for attaching molecular or cellular cargo to microtubules or motors; these strategies are discussed in Section VI. Finally, Section VII discusses higher level design considerations for motor driven devices, including ways to maximize the lifetime of motors and microtubules and approaches for introducing minute samples into these devices and detecting low levels of analyte in microfluidic channels.

II. Kinesin and microtubule cell biology and biophysics

In eukaryotic cells, organelles, vesicles, chromosomes, and protein complexes are actively transported throughout the cell by molecular motors moving along cytoskeletal

filaments. This transport system consists of both kinesin and dynein motors moving along microtubule filaments, as well as myosin motors moving along actin filaments, but members of the kinesin family carry out the bulk of intracellular transport. Conventional kinesin, the founding member of the kinesin family, serves as a model protein for understanding the molecular basis of intracellular transport, and for applications of molecular motors in nanotechnology. Conventional kinesin is a dimeric protein that contains three domains: the head or motor domain, the coiled-coil stalk that holds the two chains together, and the tail that, along with two associated light chains, is responsible for binding cargo (Figure 1).[24,25] Each motor domain contains both an ATP and a microtubule binding site, and movement is achieved by a cycle in which each head alternately binds to the microtubule, undergoes a conformational change, and releases from the track.[16,26-28] Following the discovery of conventional kinesin,[29] other members of the kinesin family were discovered based on sequence similarity in the motor head domain. The kinesin family can be divided into 14 classes based on sequence similarity and functional properties,[30] and in the human genome there are 44 kinesin genes.[31] While almost all of the application work with kinesin motors has utilized conventional kinesin, because other kinesins have different motor properties, they may become useful in future applications.

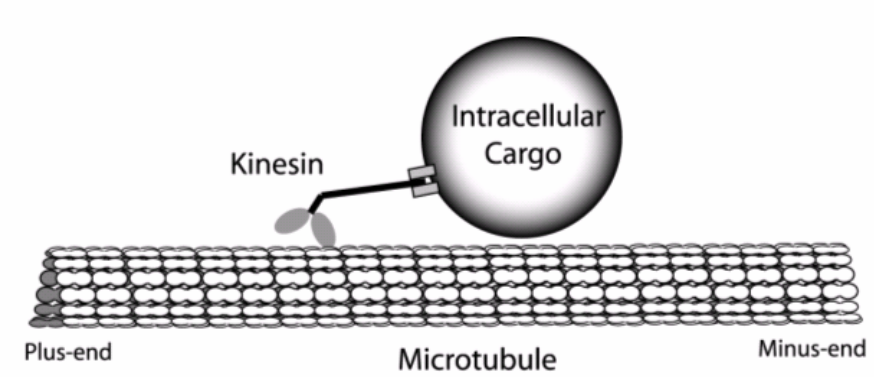

Figure 1: Structure and function of conventional kinesin. The two heads hydrolyze ATP and walk towards the plus-end of microtubules. The kinesin tail binds to intracellular cargo. Microtubules, made from tubulin subunits, are 25nm diameter and tens of microns long.

Microtubules are cylindrical polymers of the protein tubulin that are 25 nm in diameter and up to tens of microns long. 8-nm long tubulin dimers associate in a head to tail manner to make protofilaments, these protofilaments associate laterally to make sheets, and the sheets close to make hollow cylinders that normally contain 14 protofilaments.[32] Because the subunits are asymmetric, microtubules have a structural polarity – the minus- or slow growing end is

anchored near the center of the cell and the fast growing plus-ends extend to the perimeter of the cell. Experimentally, tubulin is normally isolated from cow or pig brains,[33] which are large, inexpensive and rich in tubulin owing to the neurons that require long distance intracellular transport. Microtubules can be polymerized *in vitro* from purified tubulin, covalently modified with fluorophores or other functional groups, and stabilized in polymer form with the drug taxol, making them stable for up to a week in normal buffers at room temperature.[34]

Kinesin motility assays

Nearly two decades of biochemical and biophysical experiments on conventional kinesin have resulted in a solid quantitative characterization of this molecular motor. Conventional kinesin motors walk along microtubules at speeds of nearly 1 μm/s, taking 8 nm steps and hydrolyzing 1 ATP per step.[27,35,36] The motor speed decreases approximately linearly with applied load up to a single motor stall force of 5-7 pN.[37,38] Kinesin movement *in vitro* is studied predominantly using two different assays, the microtubule gliding assay and the bead assay (Figure 2).[39,40] In the microtubule gliding assay, motors are adsorbed to glass surfaces that have been treated with the blocking protein casein, and microtubules are observed landing on and moving over the motors, analogous to a rock star being passed over the hands of an eager crowd. Typically, these assays are performed in ~20 μl flow cells constructed from a microscope slide, two pieces of double sided tape, and a cover glass, which enables facile solution exchange by simply pipeting solution in one side and wicking out the other side using filter paper or tissue.[41] Using this geometry, the motor concentration on the surface can be varied and different solutions can be introduced to optimize movement characteristics.[42] Microtubule movements are visualized by covalently labeling the microtubules with a fluorescent dye, observing them under a fluorescent microscope coupled to a sensitive CCD camera, and recording the movements on videotape or computer.[41] Because the assay is relatively easy to perform, and because the filaments are transported long distances along the surface, this geometry has generated the most attention for microscale transport applications of the kinesin-microtubule system.

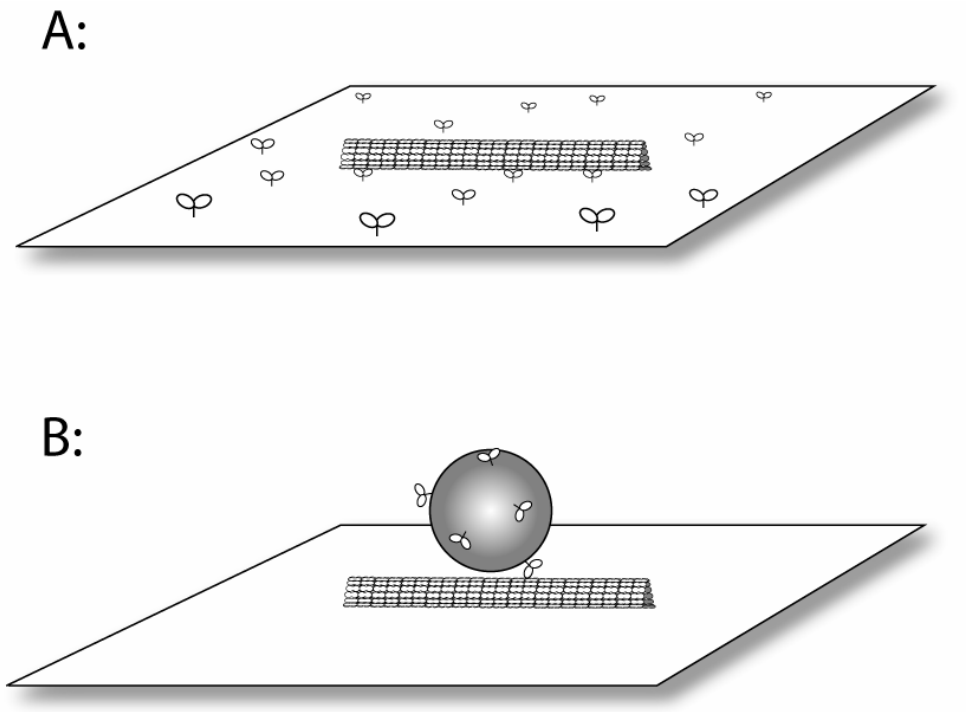

Figure 2: Assays for studying kinesin motor function. A: Schematic of the microtubule gliding assay, in which motors are adsorbed to the surface and microtubules are transported across the surface. B: The bead assay, in which microtubules are immobilized on the surface, motors are adsorbed to micron-scale beads, and the beads are transported along the immobilized microtubules.

In the other commonly used assay, the bead assay, microtubules are immobilized on glass surfaces, motors are adsorbed to micron-scale beads, and the beads are transported along the microtubules. This system is analogous to the geometry found in cells, and one advantage is that optical tweezers can be used to grab the beads and measure displacements and forces generated by the motors to nm and pN precision.[43] For transport applications, the bead can in principle be replaced by functional nanoparticles or biomolecules like proteins or nucleotides. Because the tail domain of kinesin motors can be deleted or significantly altered with no effect on the motor function,[44] in theory antibody fragments, receptors, DNA binding domains, or other protein motifs can be fused to the motor tail and these motor-cargo complexes transported along microtubules.

Motor directionality is a key consideration for applications using either the gliding assay or the bead assay. In the gliding assay, microtubules diffuse out of solution and land on the motors, and the direction of microtubule transport is defined by the orientation of the filament

(Figure 3). Because the motors, which are immobilized, move to the microtubule plus-end, the filaments move with their minus-ends leading. The coiled-coil of conventional kinesin has a region of random coil that is thought to act like a swivel, enabling the heads to rotate freely and bind to filaments only in the proper stereospecific orientation.[45] As discussed below, a large portion of the work done to harness microtubule transport for nanoscale applications has involved finding ways to control the direction of microtubule transport. The bead geometry has its own directionality problems – the transport direction is determined by the orientation of the immobilized microtubules (Figure 3). In theory, if a dense bundle of oriented microtubules could be immobilized on the surface, they would serve as ideal tracks to direct kinesin transport. However, as described in Section V below, achieving these oriented and aligned bundles has proven difficult.[9,46]

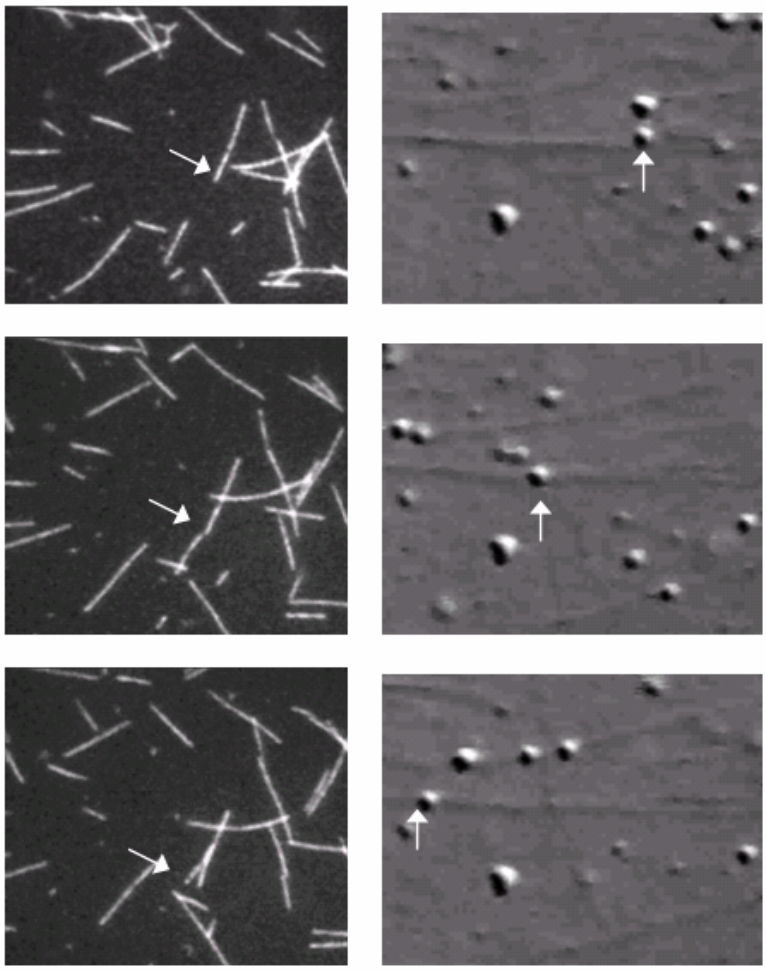

Figure 3: Kinesin-microtubule *in vitro* motility assays. Left panels are the microtubule gliding assay, showing fluorescent microtubules moving over immobilized kinesin motors. Screens are 22 μm wide and images are 2 seconds apart. As can be seen, many microtubules move over the surface, and their directions are determined by their orientation when they land on the surface. Right panels show the bead assay in which 0.2 μm diameter glass beads, to which many kinesin motors are adsorbed, are transported along surface-immobilized microtubules. Screens are 10 μm wide and images are 4 seconds apart. The direction of bead movement is determined by the orientation of the immobilized microtubules. Images at left are fluorescence and images at right are differential interference contrast microscopy.

For applications using kinesin motors and microtubules, it is important to obtain sufficient amounts of protein and keeping the proteins stable over time. As discussed above, tubulin can be purified from native sources following established protocols,[33] or it can be bought from commercial sources (Cytoskeleton Inc., Denver, CO). Conventional kinesin is generally bacterially expressed and purified,[36,44,47] but it too can be purchased. One of the hurdles to using other motors in the kinesin family is that not all of them can be bacterially expressed,[48,49] and

they can be more prone to denaturation or precipitation over time. Methods for extending the lifetime of kinesins and microtubules in engineered devices will be discussed below in Section VII. For more information and detailed protocols for kinesin and microtubule purification, and *in vitro* motility experiments, readers can consult the book Kinesin Protocols edited by Isabelle Vernos[50] or the Kinesin Home Page (http://www.proweb.org/kinesin/).

III. Theoretical transport issues for device integration

One of the main application goals for molecular motors to date has been active transport of analytes in microfluidic systems. There currently exist a number of approaches for moving fluids, analytes, and particles through microscale geometries, such as pressure-driven convective flow, electrophoresis, dielectrophoresis, and electro-osmotic flow. Rather than supplanting these methods, biomotor-driven transport should be thought of as a new approach that expands the toolbox. The best choice depends of course on the nature of the problem, and the hope is that in the future biomotor-driven transport will be combined with these other transport and separation approaches to make highly functional microscale devices.

It is informative to investigate from a theoretical perspective the range of particle sizes and transport distances where biomotor-driven transport is the most useful. Specifically, it is important consider the relative roles diffusion (Brownian motion) versus motor-driven active transport. Small analytes (proteins and nucleotides) in aqueous solutions diffuse rapidly, and any transport must overcome this diffusional mixing. For instance, as discussed below, an average protein can diffuse 1 μm in 5 msec, and even for distances of 100 μm, the average diffusion time is faster than the time it takes a kinesin motor moving at 1 μm/s to get there. It should be remembered that active transport is unidirectional, while diffusion is random, but the quantitative comparison serves as a useful guide.

Diffusion versus transport times

In Table 1, diffusion times and motor-driven transport times are compared for a range of potential analytes from proteins up to eukaryotic cells. The Einstein relation is used to derive the diffusion constant, D based on the drag coefficient of the particle γ:

$$D = \frac{k_B T}{\gamma} \qquad (1)$$

Where k_BT is Boltzman's constant (1.38 x 10^{-23} N-m) multiplied by the absolute temperature.[51] From Stokes' law, the drag coefficient of a spherical particle in low Reynold's number flow is defined as:

$$\gamma = 6\pi\eta r \qquad (2)$$

where η is the solution viscosity (0.89x10^{-3} N-s/m^2 for water at 25ºC), and r is the radius of the particle.[51,52] Combining these equations, we get the equation for the diffusion constant for spherical objects:

$$D = \frac{k_B T}{6\pi\eta r} \qquad (3)$$

For the particles in Table 1, approximate diameters are given, and diffusion constants in aqueous buffers at 25ºC are calculated.

Particle	Diameter	D ($\mu m^2/s$)	1μm		100μm		Transition Distance (μm)
			$t_{Diffusion}$ (s)	$t_{Transport}$ (s)	$t_{Diffusion}$ (s)	$t_{Transport}$ (s)	
Protein	5 nm	100	0.005	1	50	100	200
Nanoparticle	20 nm	25	0.02	1	200	100	50
Virus	100 nm	5	0.1	1	1,000	100	10
Bacteria	1 μm	0.5	1	1	10,000	100	1
Cell	20 μm	0.02	20	1	$2x10^5$	100	0.05

Notes:

$$D = \frac{k_B T}{6\pi\eta r}$$

k_BT=4.1x10^{-21} N-m @ 25ºC

Viscosity η = 0.89 cP @ 25ºC = 8.9x10^{-3} Ns/m^2

$t_{Diffusion} = x^2 / 2D$ (one dimensional)

$t_{Transport} = x / v$, where v = 1 μm/s for kinesin

Transition distance (when $t_{Transport} < t_{Diffusion}$) = $\frac{2D}{v}$

Table 1: Theoretical diffusional and transport properties for a range of biological analytes.

Using the diffusion constant, the average time it takes to diffuse a distance x in one dimension is defined as

$$t_{Diffusion} = x^2 / 2D \quad (4)$$

and the time required for kinesin-driven transport is defined as

$$t_{Transport} = x / v \quad (5)$$

where v = 1 μm/s for conventional kinesin.[36,52] In Table 1 these times are calculated for the different particles for both 1 μm and 100 μm distances. As can be seen, small particles diffuse rapidly and motor-driven transport outpaces diffusion only for distances of a few hundred microns, while large particles like bacteria and eukaryotic cells diffuse very slowly and motor transport is much faster for all distances. A helpful way to understand the utility of kinesins in microscale transport applications is to ask: at what transition distance does motor-driven transport outpace diffusion ($t_{Transport}<t_{Diffusion}$)? This distance, which gives a rough value for where motor-driven transport becomes useful, is calculated in the final column of Table 1. The important result is that for proteins and small particles like nanoparticles in the range of 10s of nm, diffusional mixing is sufficient to get particles where they need to go for distances less than a few hundred microns. However, for larger particles like bacteria and cells, diffusion is inadequate at virtually all length scales. Hence, there is significant potential for these nanoscale motors to drive and control movement of micron-scale biological objects. As discussed in Section VI, drag forces for these relatively large objects are negligible compared to the forces kinesin motors can exert.

While it is insightful to compare diffusion and transport times, it is important to appreciate that while diffusion occurs in all directions, motor-driven transport is directional. Hence, even for short distances this transport can establish and maintain concentration gradients provided the transport flux is sufficiently high. Second, because binding these small cargo pulls the particles out of solution and eliminates their random diffusional movement, it reduces the number of degrees of freedom and achieves a significant level of positional control. Furthermore, increasing the solution viscosity either by adding solutes or by creating permeable hydrogels, should be able to significantly slow diffusion rates without necessarily slowing motor transport. Finally, as the channels and sorters are fabricated using versatile photolithography techniques,[2,6,53] there is ample opportunity for optimizing the design of these systems to maximize the utility of motor-based transport and minimize diffusional mixing.

IV. Interaction of motor proteins and filaments with synthetic surfaces

A recurring technical hurdle in integrating functional proteins into engineered devices is the problem of interfacing synthetic materials with biological molecules. The problem of understanding and controlling protein-biomaterial interactions has been a major focus of the biomaterials field for decades.[54,55] For instance, in the case of implanted biomaterials the predominant approaches to maximizing biocompatibility are either to create surfaces that completely resist protein adsorption or surfaces to which proteins can adsorb but not change their activities or otherwise produce an immune response. Due of the importance of this problem, there is a body of work on protein adsorption to biomaterials. However, there are no sure fire techniques for a) creating surfaces that bind proteins but do not affect their function, or b) creating surfaces that completely resist protein adsorption.

To harness the utility of motor proteins and their cytoskeletal tracks for nanotechnology and microscale transport applications, it is crucial to control the specific adsorption of these proteins to surfaces. Here, techniques for immobilizing motor proteins and cytoskeletal filaments are reviewed.

Motor Adsorption

It can be argued that the discovery of conventional kinesin's motor activity[29] was a result of this motor's ability to bind to glass surfaces and retain its function. With proper surface passivation to reduce motor denaturation, conventional kinesin binds functionally to many types of glass as well as various oxides and other hydrophilic surfaces.[7,41,56,57] The most reliable surface passivation is pretreatment of surfaces with casein, a protein found in milk. In solution, casein forms heterogeneous aggregates with diameters on the order of 10 to 300 nm diameter.[58] The aggregation state of unproteolyzed casein is heterogeneous and depends on the Ca^{2+} concentration, the pH, the degree of phosphorylation, and other factors.[59] For kinesin experiments, commercially bought casein is dissolved in buffer and passed through a submicron filter and/or centrifuged to remove aggregates.[41]

It is clear that pretreatment of glass surfaces with casein greatly increases the activity of kinesin motors adsorbed to the surface, but the precise mechanism of action is not clear.[41] The working model is that casein aggregates 10s of nm in diameter pack on the surface, the tail domains of the kinesin motors bind between the casein particles, and the motor heads stick into solution and interact with microtubules. It cannot be ruled out that the kinesin motors bind

directly to the surface-adsorbed casein, but the fact that a great excess of casein in the motor solution does not compete with the surface-adsorbed casein (there is no reduction in the concentration of functional motors on the surface) argues against this. Also, it has been reported that casein has a chaperone-like function in stabilizing proteins against denaturation.[60] It cannot be ruled out that part of the enhancement of kinesin function by casein is due to stabilizing the motor protein structure.

One of the problems with the casein pretreatment described above is that it does not work for every motor protein. Besides conventional kinesin, there are many other kinesin motor proteins (44 total in the human genome) that vary both in their motor properties and in their intracellular cargo.[31] These different motor characteristics – direction of movement along the microtubule, affinity for the microtubule, and speed of movement – provide a rich toolbox for engineering hybrid devices based on these motors. However, while there is considerable structural consistency in the head domain, there is great divergence structurally and functionally in the tail domains.[31,61-63] The result of this diversity is that techniques for attaching one motor to a surface don't necessarily work for other motors. To date, three generalizable approaches have shown promise. The first is to adsorb antibodies to the surface and immobilize the motors through these antibodies.[64] This approach has the advantage that antibodies complementary to virtually any motor can be made, or, by attaching a universal protein tag (such as a hexa-histidine tag) to recombinant motors, one reliable antibody to be used for a range of motors. A second approach for hexa-histidine tagged motors, is to functionalize the surface with a surfactant terminated with nitrilotriacetic acid (NTA), which chelates nickel ion. His-tagged proteins bind tightly to the surface through the immobilized Ni-NTA, and the motors remain functional on these surfaces.[65] A third method is to attach motors to surfaces through biotin-avidin chemistry.[66] Streptavidin (or its equivalents avidin or neutravidin) can be directly adsorbed to surfaces or specifically bound to biotin-functionalized surfaces, and biotinylated motors can be attached to this immobilized streptavidin. Motors attached by this means have been shown to be completely functional and the bond strong enough to not be pulled off by motor forces.[67-69]

While a range of hydrophilic surfaces support kinesin-driven microtubule movements, hydrophobic surfaces do not. When glass surfaces are treated with the hydrophobic silane octadecyl trichlorethylsilane or other hydrophobic surface treatments (with contact angles >60º) prior to casein and kinesin treatment, no microtubule binding or movement is observed.[57,70] The

interpretation is that motors denature on these hydrophobic surfaces and even casein pretreatment cannot prevent this motor denaturation. This property of surface chemistry-dependent motor function has been used to define where on microfabricated surfaces microtubule will be transported and where they will not (discussed further in Section V).

Microtubule immobilization

For applications where microtubules are immobilized on surfaces and cargo-functionalized kinesins move along these filaments, a number of strategies have been developed for immobilizing microtubules. Microtubules will bind to clean glass or quartz microscope slides,[71] and this immobilization is sufficient for some investigations into motor function. However, for transport applications, a more robust immobilization is generally required. One of the most common immobilization approaches is to functionalize glass surfaces with an amino silane compound (3-Aminopropyltriethoxysilane) that confers a positive charge to the surface.[39,72] Microtubules, which have a net negative charge at physiological pH, bind tightly to these surfaces and high microtubule densities can be achieved. By lithographically patterning silanes on silicon wafers, this approach has been used to pattern immobilized microtubules at microscale dimensions.[73] Poly-lysine treated glass is another approach that uses electrostatic interactions to bind microtubules to surfaces.[74]

Two other microtubule immobilization strategies have also been shown to work well. Microtubules can be biotinylated and attached to surfaces through streptavidin, either by adsorbing streptavidin directly to the surface, or immobilizing another biotinylated protein (like biotinylated bovine serum albumin) and then using streptavidin as a glue between that protein and the biotinylated microtubule.[37,75] In principle, self assembled monolayers in which a portion of the groups are terminated with biotin could be patterned and streptavidin and microtubules patterned by the underlying biotin. Other techniques for patterning biotin or streptavidin at micron or nanoscale dimensions that have been developed for DNA microarrays or nanoscale two-dimensional protein patterns could be applied to patterning biotinylated microtubules. The final immobilization strategy, in the "turning lemons into lemonade" category, involves using dysfunctional kinesin motors to immobilize microtubules.[76,77] If kinesin protein is mishandled, for instance stored at room temperature for days, or when problems arise during expression and purification of functional motors, the motors inactivate in such a way that they bind to

microtubules but don't move along them. When these "deadheads" are adsorbed to surfaces, they act as an excellent adhesive to immobilize microtubules.

While the optimum immobilization approach depends upon the specific application, there are some general considerations that apply to all microtubule immobilization techniques. First, approaches that permit patterning microtubules on the micron or submicron scale are helpful only if filament orientation can be controlled. For instance, even with narrow strips of adhesive, microtubules can simply lay across the lines, negating the pattern. Finally, from experiences in our lab and others, it is clear that from the perspective of kinesins, not all microtubule immobilization strategies are created equal. For instance, when microtubules are immobilized through aminosilanes on glass, motors rarely interact with microtubules that are tightly adsorbed to the surface through their entire length, while they interact much more frequently with microtubules that are more loosely tacked down and have regions that are not directly attached to the surface. Whether these problems are due to deformation of the microtubules when they are tightly adsorbed or to unfavorable motor-surface interactions is not clear, but these observations emphasize that the success of any immobilization strategy must include an analysis of motor function as well.

V. Controlling the direction and distance of microscale transport

The key to harnessing the transport capabilities of molecular motors is controlling the direction of motion. For applications in microscale transport, it is this ability to direct the transport of a particle or analyte independent of fluid flows or concentration gradients that has generated the most interest in the field to date. Redirecting microtubule movements in the microtubule gliding assay has received the most attention because the filaments move long distances (detachment is not a concern), the filaments can be easily visualized by fluorescence microscopy, and they can be functionalized to transport cargo. In this section, transport applications utilizing the filament gliding will be covered first, followed by the opposite geometry, cargo-loaded motors moving along immobilized filaments.

Directing kinesin-driven microtubules

Building on the standard microtubule gliding assay, the first directed transport investigations showed that micron-scale grooves deposited on glass surfaces either by shearing PTFE or by

microfabrication guide microtubule motions parallel to the grooves.[2,4,7,78,79] This redirection arises from the nature of microtubule movements along surfaces – the front of the filament searches out new motors to bind to as it is propelled along the surface, and physical barriers that reorient this free end act to reorient the direction of filament movement (Figures 4 and 5). Building on these initial demonstrations, a number of studies using more sophisticated fabrication approaches have amply showed that microtubules can be guided and redirected using surface features. A key advance, first shown Hiratsuka et al,[6] was the demonstration that microtubules moving in microfabricated channels can be redirected by arrowhead shaped "rectifiers" built into the channels that pass filaments traveling one direction and buckle and redirect filaments traveling in the opposite direction. Later investigations extended this result using different fabrication approaches a range of rectifier shapes (Figure 6).[53,80] An important advance in this study was to identify photoresist materials that prevent adsorption of functional kinesin motors, such that when the photoresist is patterned on glass, functional motors are only found on the glass surfaces in the bottom of the channel. In both the Hiratsuka work and in a related study by Moorjani et al,[2] the key to containing the movement in the channels was not preventing protein adsorption to the walls, but rather the fact that motors that adsorbed to the photoresist denatured or otherwise inactivated. When the photoresist walls were treated to enable functional motor adsorption, the microtubules simply crawled over the walls.[2]

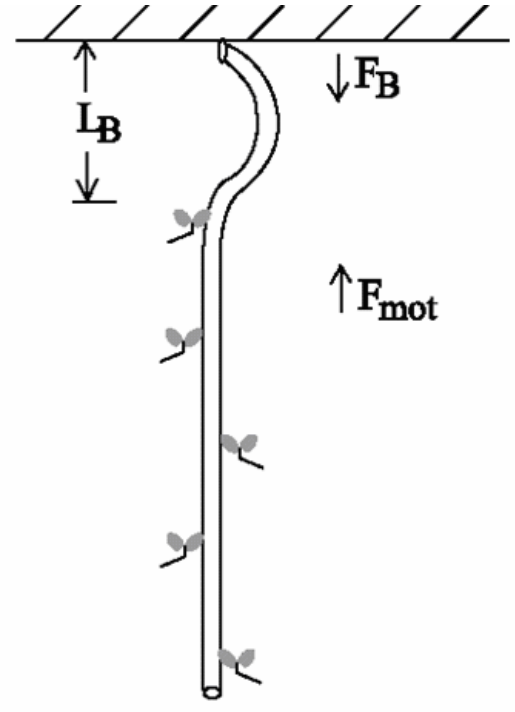

Figure 4: Physical model of a microtubule, propelled by immobilized kinesins, colliding with a wall and buckling. The length of filament that can buckle, L_B, is defined as the distance between the last motor and the wall. For the microtubule to buckle, the cumulative motor forces must be greater than the minimum buckling force for approach normal to the surface, but can be less than that if the incident angle is less than 90º. Adapted from Moorjani et al supplementary data.[2]

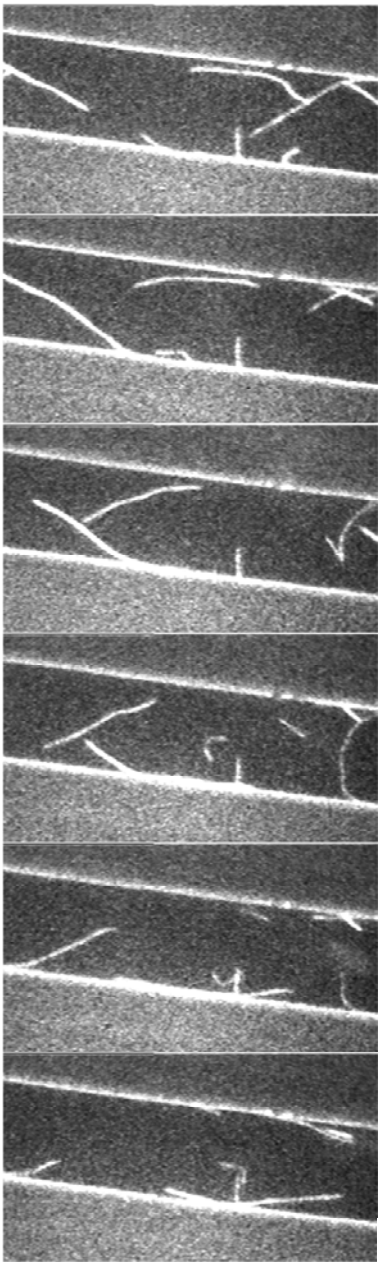

Figure 5: A microtubule being redirected by a microfabricated channel wall. The channel is glass and the walls are 1.5μm high SU-8 photoresist. The microtubule moves from left to right in the channel, bumps into the photoresist wall, and is redirected back into the channel. The Scale bar at right is 10 μm, images are 12 seconds apart. From Moorjani et al.[2]

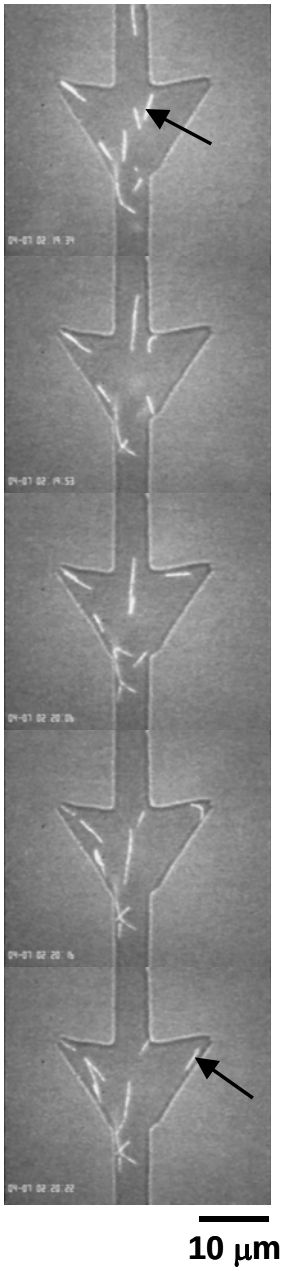

Figure 6: Arrow head shaped structures in photoresist rectify microtubule movements. In this example, 1.5μm high SU-8 photoresist walls on glass create a rectifier with inlet and outlet channels. A microtubule can be seen moving upward inside the arrowhead, bumping into the photoresist wall, and being redirected back into the channel. Rhodamine-labeled microtubules and SU-8 channel walls were imaged using simultaneous fluorescence and differential interference contrast microscopy. Figure taken from Jia at al.[53]

One goal in achieving useful transport by this approach is minimizing the number of filaments lost due to detachment from and diffusion away from the surface. If filaments are transported along the surface by a population of motors that each cyclically attach and detach from the filament, there is a low probability that all of the motors are detached at the same time, and so long transport distances can be achieved. However, when filaments are redirected by walls or crawl over walls of the channels, there can be significant filament loss if the channels are not enclosed on all sides.[2,79,81] This filament loss can be minimized by undercutting the walls of the channels to create overhangs that tend to trap the filaments in the channels.[3] Also, by designing rectifying shapes and patterning gold on the bottom of the channel to maximize motor adsorption, and silanes on the sides of the channels to resist motor adsorption, high efficiencies of filament rectification have been achieved.[80] Experiments using this "open-top" configuration where motors are adsorbed to patterned and textured surfaces and the top of the channels are left open to solution, have been instrumental in understanding the optimal channel design and surface chemistry for redirecting microtubule movements in microchannels. However, the best method for eliminating filament loss is to enclose these microchannels on all sides. For practical devices to emerge from this area of research, it is clear that enclosed microchannels will be required, together with apparatus for sample inputs and downstream detectors. As described below, there has been some initial progress toward these goals.

Movement in Enclosed Microchannels

Achieving directed movement in enclosed channels entails different design constraints than movement along topographically patterned surfaces. For instance, because microtubules are surrounded on four sides, detachment from the surface is less of a problem because subsequent rebinding is quite rapid. Furthermore, whereas guiding microtubules with open-top channels generally requires that the side walls are not populated with functional motors, in enclosed channels it is fine for microtubules to move along all four walls so long as they are moving down the channel in the desired direction. However, there are some important design constraints for creating enclosed channels for microtubule transport. First, because fluorescence microscopy is generally used to visualize microtubule movements in the channels, at least one side must be transparent, and materials that autofluoresce (which includes many photoresists) need to be avoided. Second, as described in Section VII below, PDMS, which is regularly used in

microfluidics work, can generally not be used because of its high oxygen permeability. Third, because the channels are initially filled and motor-functionalized by pressure-driven flow, the bond between the channels and the top enclosure must be sufficiently strong. Finally, the channel design must include fluidic connections for injecting motor and microtubule solutions into the channels.

Huang et al recently succeeded in achieving kinesin-driven microtubule movements in enclosed microfabricated channels.[57] A number of channel designs were investigated and a key breakthrough was creating hierarchical channels of decreasing dimensions such that solutions could be exchanged into small channels 15 μm wide and 5 μm deep without large dead volumes. The channels were constructed in SU-8 photoresist patterned on glass, covered with dry film photoresist, and topped with a silicon wafer or glass slide for mechanical stability. Microtubules moved long distances in the channels indicating that the ATP fuel was not limiting in these enclosed volumes. Furthermore, the microtubules could move upstream in convective fluid flows, which means biomotor-driven transport can move against microfluidic transport.[57] Current work in this area aims to simplify fabrication processes and optimize the channel geometries to achieve ideal microtubule transport, concentration, and redirection.

Immobilized microtubule arrays

While most studies investigating transport applications of kinesins have utilized the microtubule gliding configuration, there has also been progress on immobilizing aligned microtubules and transporting motor-functionalized cargo along these filaments. Conceptually, binding motors to analytes and transporting this cargo along immobilized filaments is the simpler geometry, and because of the ease with which motors can be engineered to contain diverse cargo binding domains in place of their tail, it has significant potential. However, the key hurdle to making this geometry work is immobilizing parallel and *uniformly oriented* filaments on surfaces. While filaments can be aligned in fluid flows, they are of mixed orientation and hence are of little use in transport applications. Current progress in obtaining arrays of uniformly oriented immobilized filaments is described below.

Brown created an array of uniformly oriented microtubules by immobilizing short microtubule seeds at defined locations, growing filaments selectively off of the plus-ends of these seeds, aligning the newly polymerized filaments by fluid flow, and then immobilizing them

on a surface (Figure 7A).[46] This process created aligned filaments, but the arrays were not the density needed for useful transport, and the method is difficult to adapt to microscale environments. Limberis used an elegant technique to bind and align microtubules.[82] Antibodies to the alpha tubulin subunit, which is exposed only at the minus-ends of microtubules and not the plus-ends, were immobilized on a surface and microtubules flowed in and allowed to bind end-on (Figure 7B). Fluid flow was then used to push the filaments over, and although they weren't immobilized in this study, this final immobilization step should not be difficult. A third technique, which may have the most potential, was demonstrated by Prots et al, who started with a field of microtubules moving over immobilized motors, aligned their direction of movement using fluid flow (over time all filaments move parallel and downstream to the flow), and then crosslinked the filaments to the motors using gluteraldehyde (Figure 7C).[83] Other experiments have shown that gluteraldehyde treatment does inhibit subsequent interactions of the microtubules with motors,[84] and the authors showed that kinesin-functionalized beads move unidirectionally along these filament arrays.[10]

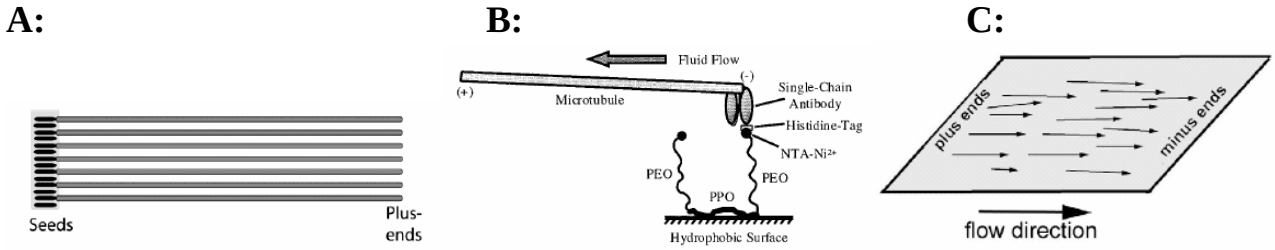

Figure 7: Three methods to create an array of uniformly oriented microtubules on a surface. A: Brown immobilized short microtubule seeds on an adhesive region, grew microtubules exclusively from the plus-ends of these filaments, and then aligned and immobilized them. B: Limberis bound antibodies for alpha tubulin to surface, captured microtubule minus-ends, and then aligned the microtubules by fluid flow. C: Prots used fluid flow to align kinesin-driven microtubules and then immobilized these filaments on the surface. Figures taken from Brown,[46] Limberis,[82] and Prots.[83]

One of the downsides of using kinesins moving over immobilized microtubules is that the transport distances are generally shorter than distances achieved by many immobilized motors moving long microtubules across surfaces. Single molecule experiments have shown that during each encounter, conventional kinesin moves approximately one micron along a filament before detaching.[39,85] These motors can then reattach and move further, but for long distance transport, longer run lengths are desired. Somewhat longer transport distances can be achieved by modifying the motors to increase the amount of positive charge in the "neck" region adjacent to the head,[86] or by binding multiple motors to the desired cargo such that dissociation of one motor

does not lead to dissociation of the entire cargo. These dissociation problems highlight the fact that if immobilized microtubules are used, they not only need to be uniformly aligned, but they need to be immobilized at high densities to maximize the rate of motor reattachment. To make this geometry feasible for analyte transport applications, more work needs to be done to develop facile methods to create high density, uniformly oriented microtubule arrays.

VI. Cargo attachment

Another important hurdle for achieving useful transport from the kinesin-microtubule system is developing methods for selective and reversible attachment of cargo to the microtubules and/or motors. For microtubules moving in microchannels, the long term vision is that the microtubules will pick up their cargo at one site and be transported along a channel, and the cargo will be deposited in a collection or analysis chamber. The question is: how are cargo attached to microtubules? Successful approaches and future ideas are reviewed here.

One problem with attaching cargo to microtubules is that, in contrast to motors, it is difficult to use the tools of molecular biology to modify tubulin sequences. Generally, tubulin for these applications is purified from cow or pig brain, and it is currently prohibitively expensive to produce genetically engineered large animals for this purpose. *In vitro* expression of recombinant tubulin has be achieved,[87] but it is technically demanding and yields are low. Hence, the primary route to modifying microtubules to enable cargo attachment has been to covalently link functional cargo attachment groups to the microtubules. By attaching reactive NHS groups to exposed amine residues on tubulin, microtubules have been covalently labeled with a number of fluorescent dyes and biotin.[34] Biotinylated microtubules have by far been the most widely used. Avidin (or its relatives streptavidin and neutravidin) contains four high affinity biotin binding sites, enabling sandwich configurations where biotin-functionalized cargo can be attached to biotinylated microtubules.[88,89]

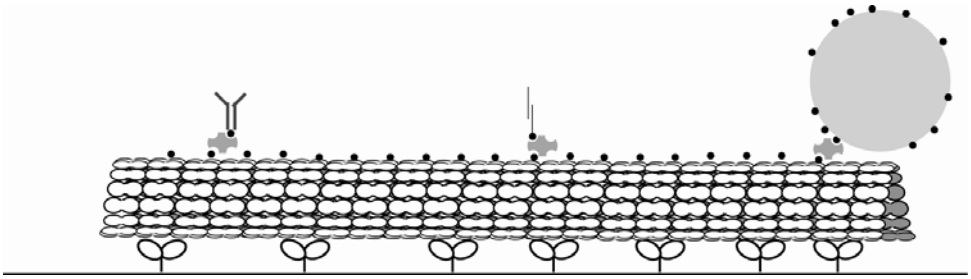

Figure 8: Attaching cargo to a microtubule through biotin-avidin chemistry. Biotin molecules (black dots), which are covalently linked to tubulin subunits bind streptavidin protein (which has four biotin binding sites). Biotin functionalized antibodies (left), DNA oligonucleotides (center), or particles (right) can be linked through the streptavidin and transported by the immobilized kinesin motors.

Proteins, single stranded DNA or RNA molecules, and cells represent three potential cargo that can be transported, separated, and/or concentrated by the kinesin-microtubule system (Figure 8). To date all three of these cargo have been attached to microtubules through biotin-avidin chemistry. Biotinylated antibodies can be purchased from commercial sources, or antibodies of interest can be biotinylated using amine-reactive biotin sold by Molecular Probes (Invitrogen Corp., Carlsbad, CA). These biotinylated antibodies can be attached to antibodies by incubating biotinylated microtubules first with neutravidin, and then with the biotinylated antibody, but care must be taken not to overload the microtubule.[89] Because the neutravidin binds to the same microtubule surface that the kinesin motors bind to, a portion of the tubulin subunits must remain unlabeled to enable motor binding. By attaching anti-GFP (green fluorescent protein) antibody to microtubules, we showed that microtubules can pick up and transport protein cargo along kinesin-functionalized surfaces.[90] This important proof of principle implies that microtubules could be modified to transport any protein for which a suitable antibody exists.

A second important cargo is nucleic acids. A possible application for a kinesin-based analytical device would be RNA analysis for examining gene expression in a small tissue sample or for detecting viruses. DNA microarrays are now widely used for expression profiling – determining what mRNA species are being expressed in a cell or tissue under certain conditions. However, if the sensitivity could be increased, smaller sample volumes could be used, ideally to the point where mRNA levels from individual cells could be analyzed. Because this enhancement would remove any signal loss due to heterogeneity between different cells in a tissue biopsy, this is an active area of research. A kinesin-based transport device would be very

valuable if it could bind selected mRNA from a cell lysate, transport these analytes down microscale channels, detect their presence, and concentrate them in a collection chamber for later analysis such as sequencing or PCR amplification (Figure 9). To demonstrate the feasibility of nucleotide transport by microtubules, Muthukrishnan et al bound single stranded DNA oligonucleotides to microtubules through biotin-avidin chemistry, and then bound fluorescently labeled complementary DNA oligonucleotides to these microtubules. Like the protein cargo-functionalized microtubules, these DNA cargo-functionalized microtubules were faithfully transported across kinesin functionalized surfaces.[88] Diez et al showed that kinesins can also be used to transport and stretch large pieces of DNA. λ-phage DNA attached to microtubules through biotin-avidin chemistry, was transported along surfaces by immobilized motors, and, when bound at one end to the surface or another microtubule, could be stretched by the motor forces up to its maximum contour length.[91]

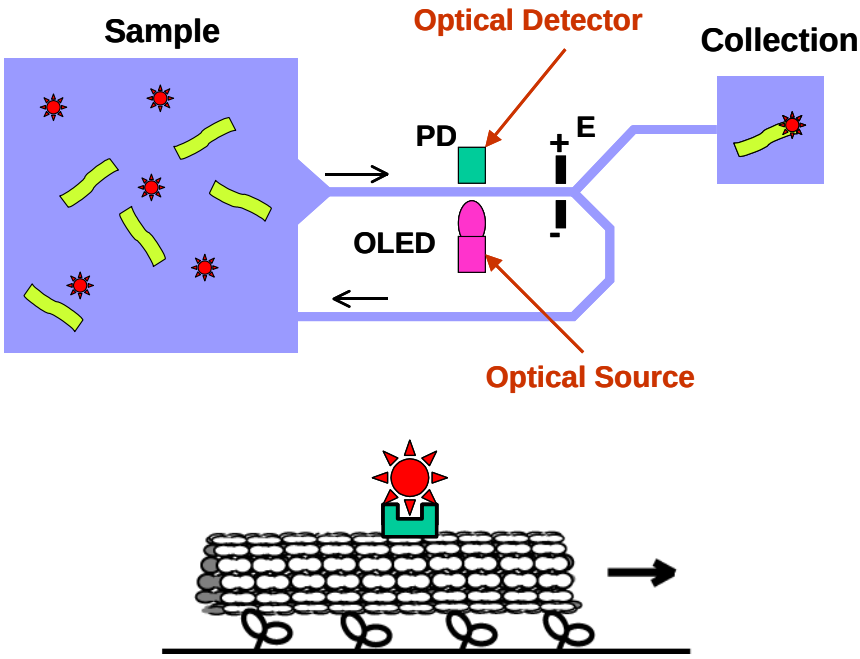

Figure 9: Diagram of motor-based detection and purification scheme. Top shows integrated system including sample chamber containing microtubules and analyte, kinesin-functionalized microchannels through which cargo is transported, LED excitation source and photodiode (PD) detector integrated into the channels, and electrodes (E) to direct cargo-laden microtubules to the collection chamber. Bottom panel shows the kinesin-based transport that will drive the system. Figure from Jia et al.[53]

Another cargo attachment strategy that has been investigated is using cyclodextrins attached to microtubules.[92] These versatile molecules are cyclic oligosaccharides that contain a hydrophobic cavity that can be engineered to bind small molecules and proteins.[93] Because they can be biotinylated, they can be attached to biotinylated microtubules through a streptavidin crosslinker and transported by surface-immobilized kinesins. Kato et al reported that cyclodextrin-functionalized microtubules moved more than 10-fold slower than normal microtubules, indicating the attachment has unfavorably altered the surface of the microtubule.[92] Hence, work needs to be done to improve this strategy, but it has the potential to be of significant utility. Other cargo-binding approaches can be envisioned such as designing RNA aptamers through directed evolution,[94] and attaching these to microtubules through biotinylated bases, but no work has been published to date in this area.

Maximum cargo size

An important question for kinesin-based transport is: what is the size limit for cargo transported by kinesins and microtubules? To investigate the potential utility of kinesins in microscale devices, Limberis and Stewart attached kinesins to 10 x 10 x 5 μm^3 silicon microchips and found the chips moved at normal motor speeds along immobilized microtubules.[56] Similarly, Jia and coworkers attached clumps of gold nanowires 6μm long to microtubules and found that they were transported at normal speeds along motor functionalized surfaces.[53] The fact that the viscous drag forces did not affect the motor transport speeds suggests that even larger objects can be transported. Below, the theoretical maximum size of an object that can be attached to a microtubule and transported by kinesins is calculated.

The theoretical size limit for spherical objects that can be attached to microtubules and transported by molecular motors can be calculated by comparing motor forces to viscous drag forces for a sphere in aqueous buffer. Individual motors can exert 5-8 pN of force.[37,38,95] Kinesin motors can be adsorbed on surfaces to densities of 1000 motors/μm^2 or more, so it is reasonable to expect a long microtubule to be able to interact with hundreds of motors.[42] If motor forces sum (which hasn't been definitively shown, but is consistent with a number of experimental observations), then it is possible that motor forces pushing a microtubule could approach 1 nN. The drag force on a particle moving in aqueous solution is equal to

$$F = \gamma v \qquad (6)$$

where γ is the drag coefficient and *v* is the velocity.[52] For a microtubule moving cargo at 1 μm/s, the drag coefficient, γ, that results in 100pN of force, which would start to slow the microtubule, is $10^{-4}\ \frac{N \cdot s}{m}$. The drag coefficient of a spherical object can be calculated from Stokes' law, given in Equation 2 above. Solving for the diameter of a sphere that would start to slow down the microtubule, the result is 1 cm! Hence, the range of objects that can in principle be transported by this system has only begun to be explored.

VII. System design consideration

The development of biomotor-driven microscale devices has evolved in three phases: a) recapitulating intracellular motility in *in vitro* experiments, b) controlling microtubule transport using microfabrication, surface processing, and other techniques, and c) creating integrated functional devices. The first phase was completed by the early 1990's. The second phase has been the focus of considerable effort over the last five years and, though it will continue to advance, the progress will likely slow somewhat. It is now that researchers are turning to the third phase, device development. There are a number of issues that need to be resolved for this work to move forward, such as protein lifetimes, sample introduction, and analyte detection and processing. Progress to date in these directions and future efforts towards these goals are reviewed here.

Protein stability and lifetime

As a tool in nanotechnology, there is a tradeoff between the exquisite functionality of biomolecular motors, and their robustness. For their small size, kinesins are able to achieve long range transport of microtubules at appreciable speeds, and generate significant forces. Because of their small size, they have the potential to be integrated into very microscale and nanoscale device geometries, enabling redundancy, multiplexing, minute sample analysis, and minimal reagent costs. However, because they are proteins, they are much more fragile than most nanoscale materials. To assess their potential utility when integrated into analytical devices, and to investigate alternate design approaches, it is necessary to determine the lifetime of kinesin motors and microtubules under normal operating conditions, and then to find conditions that extend these lifetimes.

Under normal laboratory conditions, taxol-stabilized microtubules are stable at room temperature for roughly a week,[96,97] and kinesin motors are stable on the order of hours to days depending on their concentration, storage buffer, temperature, and other variables. For experiments on motor fundamentals, as long as the motors are stable for a working day, there is no need to put the work in to extend their lifetime because each morning a new aliquot of protein can be thawed from the -80ºC freezer. Hence, it has been the interest in device integration that has driven experiments to uncover the mechanisms that limit the working lifetimes of kinesin and microtubules, and to extend these lifetimes.

In the kinesin-microtubule field the most convenient technique for observing motor function fluorescence microscopy. Microtubules can be visualized by differential interference microscopy, but for applications where motors and/or cargo are to be observed fluorescence is much more versatile. In the microfluidics field, a standard approach for creating channels has been PDMS molding and bonding to glass,[98] and in a perfect world these two approaches could be combined. However, workers studying fluorescent microtubule movements in PDMS channels quickly ran into troubles with photodamage.[99] In the standard microtubule gliding assay, the microtubule cocktail includes buffering salts, ATP to drive motility, and an antifade consisting of an oxygen scavenging system and reducing agent.[41,45] In the absence of antifade, exposure of the fluorescent microtubules to the high intensity mercury arc lamp used for fluorescence excitation results in rapid photobleaching, loss of movement, and microtubule depolymerization due to oxygen free radical damage. With antifade, the movement of rhodamine-labeled microtubules can be visualized for roughly 10 minutes under continual high intensity illumination. Without illumination or by shuttering the light, microtubule movement lasts for ~10 hours or longer.[100] However, when the flow cell is enclosed with PDMS instead of glass, illumination leads to inhibition of motion and microtubule breakage or depolymerization after approximately one minute.[99,100] The cause of failure was traced to the high oxygen permeability of this silicone polymer, which overwhelms the antifade's ability to remove all of the soluble oxygen. PMMA enclosures also showed this same property. While these results don't rule out these materials as device components, if they are to be used they must be combined with another barrier to oxygen diffusion, increasing the complexity of fabrication.

A central concern in the feasibility of kinesin-microtubule powered devices is long term storage: if hybrid biological/synthetic devices are manufactured, how long can they be preserved

before use? Interestingly, this is a similar concern in the field of tissue engineering, where products like artificial skin that include live cells integrated into a polymer scaffold must be made at a manufacturing site, stored and shipped, and then unpackaged and used by a doctor. The obvious first approach is freezing; kinesin motors, tubulin, and even taxol-stabilized microtubules can be frozen on liquid nitrogen and stored at -80ºC for years with little loss in functionality. In theory, hybrid devices could be simply dunked in liquid nitrogen and thawed before use, but there are a number of possible problems with this approach, including mechanical problems due to unequal expansion coefficients of the various materials, and the requirement for a deep freeze at every point of use.

There has been some recent work on novel approaches to extending kinesin and microtubule lifetimes, which have yielded interesting results. With the goal of identifying materials processing approaches that could be used to created hybrid devices, Verma et al tested the stability of casein, kinesin motors, and microtubules when exposed to various solvents and strippers used in materials processing and nanofabrication. Interestingly, kinesin motors survived when exposed to either pure isopropyl alcohol or acetone, two solvents commonly used to remove photoresist.[101] Microtubules were less robust than motors, but they did survive exposure to 1:4 dilutions of these chemicals in aqueous buffer, and these diluted solvents retained their ability to strip photoresist. While not directly impacting device lifetime, these studies provide new approaches for device manufacture and they suggest that these proteins are more stable than may be indicated by their lifetimes in buffer at room temperature. In other work, it was found that drying immobilized kinesins by wicking the solution out of flow cells and storing them under different conditions resulted in longer kinesin lifetimes.[102] Hence, it is possible that instead of freezing hybrid devices, simply desiccating them and then reconstituting them may provide a simple alternative. These findings should lessen the constraints to hybrid device design.

Sample introduction and detection

Two future hurdles to creating kinesin-driven analytical devices are sample introduction and analyte detection. To date there is scant work in this area, but because these requirements are universal to virtually all sensor systems, ideas and modules will undoubtedly be borrowed from other disciplines. With respect to sample introduction, one design constraint is that these

biomotors work in aqueous environments. As discussed above, kinesins can survive being dried or exposed to solvents, but for their proper function they must remain in proper buffer solutions. Hence, to detect airborne particles (viruses or bacteria for instance), the samples would need to be concentrated (i.e. filtered) from the atmosphere, and then transferred to the aqueous state. While not impossible, there are clearly a number of engineering problems to be solved for this application to become feasible.

An area where the strengths of kinesin-powered devices are maximized is the detection of proteins, RNA, or small molecules in minute aqueous samples. As discussed above, detection of specific molecules from single cells is a holy grail of sorts for bioanalytical detection. Delivering the contents of single cells to kinesin-powered transport and detection machinery is a task in itself. Both convective flows and dielectrophoresis have been used to transport cells in microfluidic systems.[103,104] Additionally, both optical and electrical methods have been developed to rapidly lyse cells, enabling their intracellular contents to be delivered to the microanalysis machinery.[105,106]

Analyte Detection and Collection

As in all analytical systems, analyte detection will be a key component to future kinesin-powered hybrid devices. The first choice for analyte detection is fluorescence, because it is sensitive and is the standard technique for observing microtubules moving in channels. Building on work guiding microtubule transport in microfluidic channels, analyte detection could be achieved by binding the analyte to the microtubule and observing the change in fluorescence. If the analyte (cell, protein, or nucleotide) is fluorescent and has a different emission peak than the fluorophore labeling the microtubule, analytes could be detected by observing the microtubule fluorescence at one wavelength and measuring the analyte fluorescence at a second wavelength. In this case the microtubule acts to both concentrate and to localize the analyte.

While this detection could be achieved using epifluorescence microscopy, there is potentially for achieving much more utility and sensitivity by integrating the optical components into the microfluidic chips containing the motors and analyte. For instance, if an LED and photodiode are integrated into the microchannel containing the microtubule and bound analyte, the detector could be within microns of the sample (Figure 9). This placement should maximize photon capture and reduce background signal due to extraneous signal. One advantage of using

the kinesin-microtubule transport system for analyte detection is that the movement speed is stereotyped, so for low signal levels the time average of fluorescence as the analyte moves across the detector could be used to increase the signal-to-noise ratio.

VIII. Conclusion

Because of the highly evolved properties of protein machines, there is incredible potential in integrating them into hybrid biological/synthetic devices. Their nanometer size scales match well with emerging nanofabrication capabilities and novel functional nanoscale materials. A continuing struggle in nanotechnology is finding techniques to manipulate and organize molecules and nanoparticles in cases where self-assembly is not energetically favorable. Because biomolecular motors have evolved to transport and organize biological materials on the nanoscale and microscale in cells, they have the potential to solve a number of these general problems in nanotechnology. This chapter has reviewed the *in vitro* transport capabilities of kinesin motors, with an eye toward creating microfluidic devices for analyte detection. This application is one of the first envisioned for these particular protein machines, and it is anticipated that as new approaches are developed for to gain ever more control over the kinesin-microtubule system that these biological machines will be applied to other nanoscale systems.

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