Micro- and Nanofabrication Processes for Hybrid Synthetic and Biological System Fabrication

Vivek Verma, William O. Hancock, and Jeffrey M. Catchmark

***Abstract*—The application of micro- and nanofabrication processes to the development of hybrid synthetic and biological systems may enable the production of new devices such as controllable transporters, gears, levers, micropumps, or microgenerators powered by biological molecular motors. However, engineering these hybrid devices requires fabrication processes that are compatible with biological materials such as kinesin motor proteins and microtubules. In this paper, the effects of micro- and nanofabrication processing chemicals and resists on the functionality of casein, kinesin, and microtubule proteins are systematically examined to address the important missing link of the biocompatibility of micro- and nanofabrication processes needed to realize hybrid system fabrication. It is found that both casein, which is used to prevent motor denaturation on surfaces, and kinesin motors are surprisingly tolerant of most of the processing chemicals examined. Microtubules, however, are much more sensitive. Exposure to the processing chemicals leads to depolymerization, which is partially attributed to the pH of the solutions examined. When the chemicals were diluted in aqueous buffers, a subset of them no longer depolymerized microtubules and in their diluted forms still worked as resist removers. This approach broadens the application of micro- and nanofabrication processes to hybrid synthetic and biological system fabrication.**

***Index Terms*—Biocompatibility, biological systems, casein, hybrid systems, kinesin, microfabrication, microtubules, nanofabrication, polymethylmethacrylate (PMMA) lithography, protein patterning.**

I. Introduction

MICRO- and nanofabrication processes can create devices and structures with dimensions ranging from micrometers to less than 10 nanometers. Absent are motor technologies based on silicon or related materials that can power such devices, directing attention toward hybrid systems integrating biological motors [1]–[3]. One approach toward the production of molecular motor-powered micro- and nanoscale devices is to develop nanofabrication processes that enable patterning of motor proteins without impacting their functionality. Although nanofabrication technologies provide the ability to create structures whose dimensions are comparable to those of motor proteins and microtubules, the particular lithographic and material etching processes involved in traditional device fabrication employ chemistries that are considered incompatible with the incorporation of biological materials [4]. In order to develop a set of "design rules" for hybrid system fabrication, we have examined the effect of micro- and nanofabrication resists, developers and removers on the functionality of the surface blocking protein casein, the motor protein kinesin, and microtubules using the standard microtubule gliding assay. Lithography resists examined in this study were polymethylmethacrylate (PMMA), (Microchem Corp.), an electron beam sensitive resist, and UV5 (Shipley Corp.), a deep ultraviolet light-sensitive resist. The resist developers methyl iso-butyl ketone (MIBK, Mallinckrodt Baker Inc.) and tetramethylammonium hydroxide (TMAH, Microposit MF-CD-26, Rohm & Haas Electronic Materials LLC) were also investigated, as well as the removers acetone and isopropyl alcohol (IPA).

A. Motor Proteins and Microtubules

Biological motors such as kinesin and dynein transport intracellular cargo and position organelles in eukaryotic cells via unidirectional movement along cytoskeletal tracks called microtubules [5]–[7]. This process is powered by the conversion of chemical energy to mechanical work via the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP) [7]. Conventional kinesin contain two domains with dimensions measuring $7 \times 4 \times 4$ nm [6], [7] that "walk" in a coordinated manner along microtubule tracks, while the motor's tail acts as an attachment domain to bind intracellular cargo. Microtubules are cylindrical polymers of the protein tubulin that are 25 nm in diameter and up to tens of micrometers long, and have an inherent structural polarity [8]. Conventional kinesin moves toward the fast growing or plus-end of microtubules, while other motors like dynein move toward the minus-end, thus, establishing a two-way transport system within cells. The preferred direction of polymerization of microtubules and unidirectional movement of motor proteins along microtubules create unique challenges for hybrid system development. In particular, orienting microtubules is especially difficult. Apart from patterning the microtubule seed in specific locations, the polymerizing microtubule must remain confined in the desirable direction. However, as microtubules polymerize, they tend to lose their linearity and form a mesh (refer to Brown *et al.*), making polymerizing microtubules in preferred directions difficult.

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V. Verma is with the Department of Engineering Science and Mechanics, Pennsylvania State University, University Park, PA 16802 USA.

W. O. Hancock is with the Bioengineering Department, Pennsylvania State University, University Park, PA 16802 USA.

J. M. Catchmark is with the Nanofabrication Facility and Department of Engineering Science and Mechanics, Pennsylvania State University, University Park, PA 16802 USA (e-mail: jcatchmark@engr.psu.edu).

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B. Traditional Micro- and Nanofabrication Processing

Processes used to fabricate micro- and nanoscale structures include lithography, etching, and deposition, which can be performed on a variety of substrates including silicon and glass [9]. Deposition of materials onto substrates can be accomplished using a number of processes including simple spin-on application of liquid materials and cure, plasma sputtering, evaporation and chemical vapor deposition (CVD). However, plasmas are inherently destructive to biological materials since they contain sufficient energy to ionize gasses. Similarly, CVD processes implement temperatures from 350 °C to over 600 °C, and spin-on materials that are intended to be permanent are typically cured at temperatures ranging from 200 °C to over 500 °C for extended periods of time. Proteins denature at these high temperatures, limiting the utility of these processes for creating hybrid synthetic/biological materials. Sacrificial spin-on materials such as resists can often be cured at temperatures less than 200 °C, so these materials are examined in the present study. Motor proteins in particular, however, cannot withstand temperatures in excess of 45 °C [10] limiting the biocompatibility of these deposition processes.

Materials can be selectively removed using wet chemical etching, ion beam milling, or reactive ion etching (RIE). RIE uses an ionized gas plasma to remove material both chemically and mechanically, and ion beam milling is a completely physical removal process using accelerated ions such as argon. Both of these processes are destructive to biological materials. Wet chemical etching is performed with solutions whose composition is typically either acidic or basic, also posing hazards to biological materials.

Protein patterning requires forming defined regions where protein molecules can bind and maintain a desired functionality. In order to evade the use of removal process such as RIE, ion beam milling, and chemical etching, which are destructive to biological materials, other lithography processes were examined. Lithography processes, including electron beam, optical projection, scanning probe, and embossing are used to pattern materials at the micro- and nanoscale [9], [11]–[14]. Optical projection lithography is the most commonly used patterning process for integrated circuit and microelectromechanical systems (MEMS) fabrication and can achieve feature dimensions less than 100 nm. This process involves the projection of ultraviolet (UV) light through a patterned mask to selectively expose different regions of the surface of a substrate that has been coated with a UV sensitive organic polymer resist. The exposed (or unexposed) regions of the resist are removed using a wet developer. Features with dimensions less than the 25-nm diameter of a microtubule can be patterned using electron beam lithography and an electron beam sensitive resist. However, the photosensitive and electron beam sensitive resists, developers, and removers can all potentially interfere with the functionality of biological materials. In this paper, we examine the effects of common photo and electron beam sensitive resists and their respective development and removal chemistries on casein, kinesin, and microtubule proteins.

C. Hybrid System Integration

Individual kinesin molecules can exert maximal forces on the order of 6 pN [6], which is sufficient to transport intracellular cargo. Kinesin can be surface immobilized with a packing density of approximately 10^3 motors per μm^2[18], giving theoretical cumulative forces in the order of nano-Newtons per micrometer squared. The current thrust is to utilize the kinesin microtubule system to power hybrid devices such as transporters, gears, levers, micropumps, microgenerators, or other MEMS/nanoelectromechanical ssystems (NEMS) devices. The key to producing such hybrid systems lies in the application of established micro- and nanofabrication processes to the organized arrangement of motor proteins and microtubules on surfaces. To achieve this goal, two device architectures have been explored to date. The first, termed the microtubule gliding assay, involves immobilizing motors to transport cargo-laden microtubules [19]–[22] [see Fig. 1(a)]. The second approach utilizes immobilized microtubules to transport cargo-laden kinesin motors [23], [24] [see Fig. 1(b)]. To derive useful work from the microtubule gliding assay, it is necessary to control the direction of microtubule movement. In an early study, Dennis *et al.* [25] used shear-deposited poly (tetrafluoroethylene) (PTFE) film to induce microtubules to move preferentially along one axis. More recently, Hiratsuka [19] fabricated arrowheads of various shapes using photoresist on glass. Because motility was achieved only on the glass surface, and the photoresist walls redirected microtubule movement, unidirectional microtubule movement was achieved. Moorjani [21] used SU-8 channels to direct microtubule movement. Microtubules moving on a glass surface were redirected when they encountered the SU-8 sidewall. They obtained differential motility on glass and not on SU-8, but there was nonspecific binding of kinesin motors on SU-8 surface.

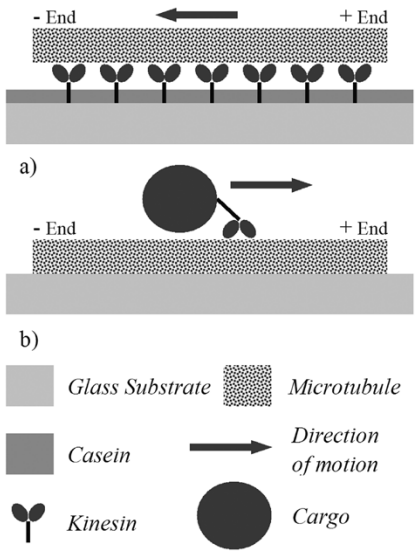

Fig. 1. (a) A microtubule moving toward its minus end via surface immobilized kinesin, and (b) a kinesin bound to cargo moving along a microtubule toward its plus end.

The aforementioned studies involved adsorbing motors to engineered surfaces, but to expand the range of geometries and applications for biological motors, there is a need for new processing approaches for patterning functional motors and microtubules. For instance, Ilic [4] used parylene as a dry lift-off material to pattern proteins on various substrates, achieving

minimum sizes of 2 μm. Unfortunately, this method imposes limitations on the geometry of the patterned area, since the peeled-off film must be contiguous and the features designed for mechanical stability of the film during the peel-off process. Other approaches to protein patterning to date include patterned surface functionalization through photoresist or via the activation or deactivation of UV-sensitive molecules using lithography [26]. Earlier work was performed to create patterns of rabbit IgG using novel photoresists [27], [28]. However, this process used a highly basic resist TMAH which, as described below, is lethal to kinesin and microtubules even in diluted forms. Ward *et al.* [29] introduced a UV polymerization technique that created a patterned substrate onto which proteins can be deposited but were not subsequently patterned. Bachand *et al.* [30] selectively bound F_1-ATPase motors onto patterned nickel features. Mahanivong *et al.* [31] fabricated channels using laser ablation of a photosensitive polymer containing O-acryloyloxime acetophenone oxime (AAPO) deposited onto bovine serum albumin (BSA) blocked glass slides. This process enabled the patterning of myosin into channels but nonspecific binding of actin filaments was observed. Finally, Brunner *et al.* [32] studied the compatibility of widely used polymers on the stability of kinesin and microtubules but did not explore the patterning of polymers or kinesin. In our paper, we examine the applicability of conventional micro- and nanofabrication resists and processes for the patterning of kinesin and related proteins. Our goal is to expand this toolbox of fabrication approaches for patterning motor proteins and their microtubule tracks with the eventual goal of creating new hybrid devices with engineered functionality.

II. Materials and Methods

A. Kinesin Motors and Microtubules

Full length *Drosophila* kinesin heavy chain kinesin was bacterially expressed and purified by nickel column chromatography as reported previously [18], and the motor concentration estimated from gel densitometry on comassie-stained gels. Kinesin solutions consisted of 4-μg/mL kinesin, 0.2-mg/mL casein and 100-μM MgATP in BRB80 buffer (80-mM PIPES, 1-mM $MgCl_2$, and 1-mM EGTA, pH 6.9). Bovine brain tubulin was purified and fluorescently labeled with rhodamine as previously described [33], [34]. To polymerize microtubules, 4-mg/mL tubulin, 1-mM GTP, 4-mM $MgCl_2$ and 5% DMSO were combined in BRB80 buffer, and the temperature was raised to 37 °C for 20 min. Polymerized microtubules were then diluted 100-fold in BRB80 plus 10-μM paclitaxel to stabilize them, resulting in a population of microtubules with lengths in the range of 5–20 μm. Motility solution was made as described in [18]. Casein protein (Sigma, Inc.) was dissolved in BRB80 buffer and centrifuged and filtered to remove insoluble components.

B. Flow Cell

The flow cells used in these experiments (Fig. 2) were constructed by applying strips of two-sided cellophane tape to the edge regions of a microscope slide (Fisher Finest Premium Microscope slides) and then covered with glass cover slip (Corning 1 1/2, 18-mm square) forming a chamber. The volume enclosed between microscope slide and the cover slip was $\sim$ 20 μL.

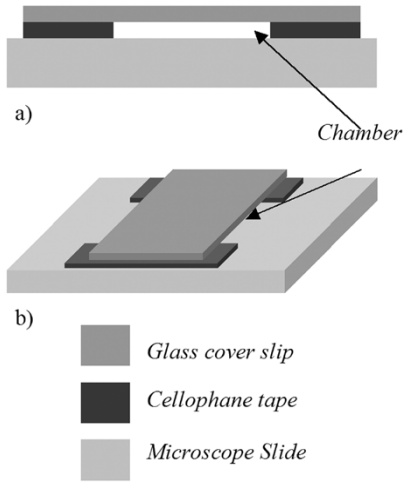

Fig. 2. (a) Cross sectional and (b) perspective views of the flow cell.

C. Standard Motility Assay

The basic process for performing the standard motility assay is shown in Fig. 3. Variations on the standard motility assay were used to quantitatively assess the impact of micro- and nanofabrication processing chemicals and resists on the functionality of casein, kinesin, and microtubules. The standard assay consists of the following steps. First, glass cover slips are incubated with 0.5 mg/mL casein for 5 min. This treatment partially blocks the surface, preventing binding and denaturation of the kinesin motor domain, while retaining the ability of the kinesin tail to bind to the surface. Applying casein also prevents nonspecific adsorption of microtubules to the surface. Next, the glass cover slips are incubated with kinesin motors for five minutes to adsorb the motor tails to the surface. Finally, microtubules are passed into the flow cell, and after an incubation period of 10 minutes, visualized by epifluorescence microscopy (Nikon E600, 100 $\times$, 1.3 N. A. Plan Fluor objective). Depending on the test requirements, we either pretreated the glass cover slips with a series of chemicals or injected chemicals into the flow cell during the motility experiment to test their compatibility with the proteins. The impact of these chemicals was quantified by comparing the density of microtubules bound to the surface-adsorbed kinesin motors to that seen in the control experiment using an untreated coverslip. Specifically, the average number of microtubules seen in a 35×35 μm area whose length was greater than 3 μm and were moving was counted for five different areas within a given flow cell chamber.

III. Results and Discussion

In these studies, the standard motility assay was modified to quantify the impact of temperature, lithography resists, ultraviolet light, developers, and removers on the functionality of the protein casein, kinesin motor proteins, and microtubules. In particular, the resists PMMA (Shipley Corp.) and UV5 (Shipley Corp.), their development chemicals TMAH (tetramethylammonium hydroxide) and MIBK (methyl iso-butyl ketone), and their removers acetone and IPA (isopropyl alcohol) were examined. PMMA and UV5 are common positive electron beam (e-beam) and deep ultraviolet-sensitive resists, respectively, and

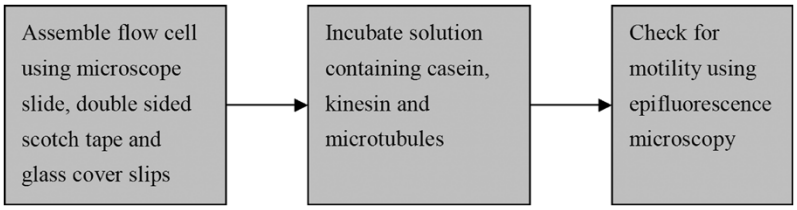

Fig. 3. Basic procedure for performing a standard motility assay.

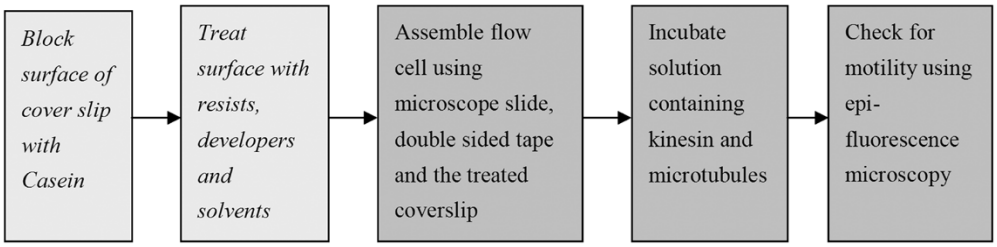

Fig. 4. Modified procedure for studying the impact of surface treatment on casein. The text in italic indicates where the standard assay has been modified.

are used for defining submicrometer patterns. These resists are spin coated on substrates, thermally cured, and then exposed to electron beam or optical radiation to chemically modify their polymeric structure, enabling them to be selectively dissolved in a developer. PMMA is developed in a mixture of MIBK and IPA, while UV5 is developed in TMAH (specifically 1:1 TMAH: deionized water). Acetone is used, in conjunction with IPA, for removing the unwanted resist after subsequent patterning processes and for cleaning the wafer surface.

The results are organized as follows. First, the influence of surface cleaning procedures is examined, followed by examinations of the effects of chemicals on casein, kinesin, and microtubules. Finally, the motility on selected photoresists and the influence of UV light on kinesin function are examined.

A. Motility Tests on Treated Glass Slide Surfaces

In practical micro- and nanofabrication processing, substrate surfaces are routinely exposed to cleaning solvents or developers. To determine if this surface pretreatment impacts kinesin-based motility, four glass cover slips (Corning 1 1/2, 18-mm square) were submerged into either TMAH, MIBK, acetone, or IPA for 10 min. These cover slips were then used to perform the standard motility assay and the result compared to a control unprocessed cover slip. In all cases, microtubule motility was observed on the preexposed surfaces, and the number and movement behavior of the microtubules did not differ from control. This result indicates that any surface modification produced by these chemicals did not affect the ability of casein to block the surface or support the functional behavior of the kinesin motors.

B. Effects of Chemicals on Casein

Casein is applied to glass surfaces to prevent the active motor domain of the kinesin protein from binding to the surface and denaturing. if no casein treatment is used in these experiments, no motility is observed, so if the kinesin motors need to survive the processing steps, then the casein functionality must survive as well. Hence, the effect of temperature and exposure to PMMA, UV5, MIBK, TMAH, acetone, and IPA on casein was examined using the modified motility assay shown in Fig. 4.

TABLE I
RESULTS OF EFFECT OF TEMPERATURE ON CASEIN. NUMBER OF DATA POINTS $(N) = 5$ FULL SCREENS. FULL SCREEN $= 57 \times 70\ \mu$m. KEY FOR THE TABLE: 1–6 LOW, 7–14 INTERMEDIATE, 15–20 HIGH

Bake time in minutes	Casein added to incubation solution	Number of microtubules	Density of Microtubules
Control	Yes	20.0 ± 2.10	High
0	No	8.0 ± 1.90	Intermediate
2	No	7.4 ± 1.50	Intermediate
5	No	7.0 ± 1.26	Intermediate
5	Yes	20.0 ± 1.41	High

Temperature is an important process parameter since lithography resists typically require one or more curing steps. To examine the effect of heat treatment on casein, several experiments were performed. Five cover slips were coated with casein. One of these cover slips was baked on a hot plate at 180 °C for 2 min and two cover slips were baked at 180 °C for 5 min. The temperature of 180 °C was chosen because it is the typical cure temperature for PMMA, and most other resists such as UV5 require lower cure temperatures. One of the unbaked casein-coated cover slips was used to perform the standard motility assay as a control. The other cover slips were used to construct flow cells for modified motility assays. Kinesin motors were flowed into the chambers and incubated for 5 min, and microtubules were then flowed into the chamber and motility was measured. In all but one of these assays, where the second casein coverslip was baked for 5 min, casein was not added to the kinesin and microtubule solution as done in the standard motility assay, ensuring that untreated casein would not form an additional blocking layer on top of the previously deposited casein that had been exposed to elevated temperatures. The results of these studies are shown in Table I.

Fig. 5. Motility assay on acetone treated casein at $t = 0$, 11, and 22 s. Screen dimensions are 35×35 μm.

Motility was observed on heat-treated casein, but the density of microtubules seen was not as high as the density observed in the control assay. However, the same density of microtubules was also observed in the case where no heat treatment was performed, but casein was not included in the incubation solution. This result suggests that the heat treatment is not having a significant effect on the functionality of casein, but rather the microtubule density has been impacted by the removal of the casein in the incubation step, which was not done in the standard motility assay (control) or in one of the examined cases where the casein had been baked for 5 min at 180 °C (see Table I). In both of these cases, we observed a high density of microtubules. This intriguing result suggests that casein and kinesin are engaging in a dynamic coassembly process to form an active layer of functional motor proteins on the surface of the glass slide, which cannot be achieved via a serial process involving first coating the surface with casein and then coating the surface with kinesin.

Next, the effects of MIBK, TMAH, acetone, and IPA on casein were studied. Four glass cover slips were coated with casein, baked on a hot plate at 180 °C for 2 min, and then submerged into one of these chemicals for 10 min. Flow cells were then constructed using the treated cover slips, the incubation solutions containing kinesin and microtubules without casein were introduced as described above, and motility was examined. An intermediate level of motility was observed for all cases except for TMAH, demonstrating that the surface-adsorbed casein can tolerate MIBK, acetone, and IPA. The results are summarized in Table II, and three screen shots showing the movement of microtubules on acetone-treated casein are shown in Fig. 5. The low density of microtubules observed in the TMAH case is attributed to the high pH of TMAH, which was measured to be 13.6 using a Corning 440 pH meter.

The next question was whether deposition of a resist on top of surface-adsorbed casein and then removal of that resist impacts casein function. To examine the resists PMMA and UV5, casein was incubated on two glass cover slips, air dried, and heated on a hot plate at 180 °C for 2 min. The coverslips were then coated with either PMMA or UV5. PMMA was spin-coated at 3000 r/min for 40 s and baked for 90 s at 180 °C, while UV5 was spin-coated at 4000 r/min for 40 s and baked at 135 °C for 1 min. After baking, the resists were stripped using acetone, and the glass surface was cleaned using IPA. Two flow cells were constructed using these cover slips, casein-free kinesin and microtubules were injected into the flow cells, and motility was examined. The results are contained in Table II. Again, an intermediate level of motility was observed. This result indicates that PMMA and UV5 can be coated on top of casein and stripped off without affecting casein's functionality. Three screen shots showing the movement of microtubules on PMMA-treated casein are shown in Fig. 6.

TABLE II
RESULTS OF TREATMENT OF CASEIN WITH DIFFERENT FABRICATION CHEMICALS. $N = 5$ FULL SCREENS

Chemicals		pH	Number of microtubules	Density of Microtubules
Control		6.9	20.0 ± 2.10	High
Removers/ Solvents	IPA	5.85	8.2 ± 1.33	Intermediate
	Acetone	7.71	7.2 ± 1.60	Intermediate
Developers	MIBK	6.16	10.6 ± 1.62	Intermediate
	TMAH	13.6	1.4 ± 1.02	Low
Resists	UV5	-	10.8± 2.32	Intermediate
	PMMA	-	7.6 ± 1.36	Intermediate

C. Effect of Chemicals on Kinesin

To investigate the effect of developers and strippers on kinesin motor proteins, casein and kinesin were incubated in four flow cells using the standard procedure. MIBK, TMAH, IPA, and acetone were then separately flowed into one of the four flow cells and left undisturbed for 10 min, followed by injection of microtubules to study motility. The experimental protocol is described pictorially in Fig. 7.

The results of motility experiments on treated kinesin are summarized in Table III. Again, medium to high levels of motility were observed in all cases except for TMAH, where the high pH of TMAH presumably denatures the kinesin motors. The effect of lithography resists on kinesin motors was not tested because of the post application bake step of resists that exceed 45 °C.

The impact of dissolved PMMA and UV5 resists on kinesin was also examined. This is particularly important because direct patterning of motor proteins using resist-based lithography will require the motors to withstand a resist removal process step. To investigate this, Scotch Tape was used to cover parts of two microscope slides, the resists were spin coated onto the slides, and then the tape was removed to produce a region on the slide without any resist. After removing the tape, the slide coated with PMMA was baked for 90 s at 180 °C, and the slide coated with

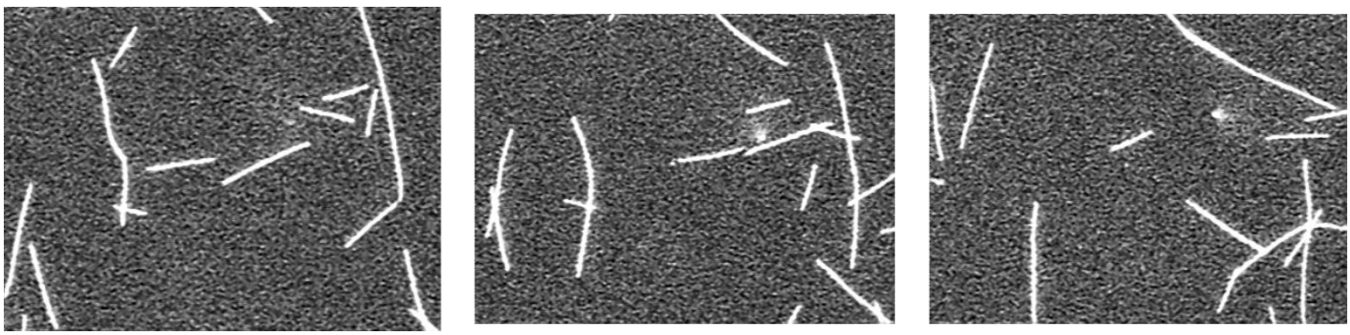

Fig. 6. Motility assay on PMMA-treated casein (t = 0, 20, and 60 s). Screen dimensions are 35×35 μm.

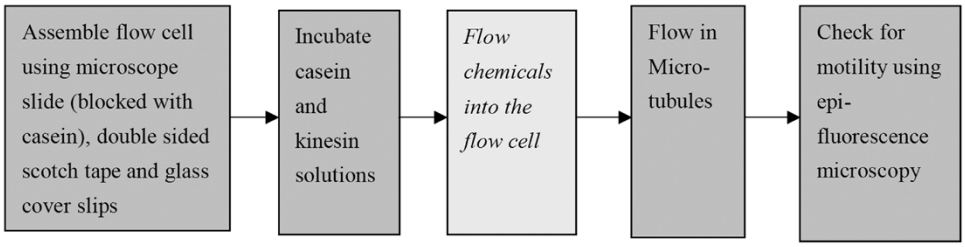

Fig. 7. Modified motility assay to test the effect of chemicals on kinesin.

TABLE III
RESULTS OF TREATMENT OF KINESIN WITH DIFFERENT FABRICATION CHEMICALS. N = 5 FULL SCREEN SHOTS

Chemicals		pH	Velocity (μm/s)
Control		6.9	0.8 ± 0.05
Removers/ Solvents	IPA	5.85	0.66 ± 0,04
	Acetone	7.71	0.61 ± 0.09
Developers	MIBK	6.16	0.67 ± 0.02
	TMAH	13.6	0.0
Resists	PMMA	—	0.79 ± 0.04
	UV5	—	0.79 ± 0.05

TABLE IV
RESULTS OF TREATMENT OF KINESIN WITH DIFFERENT FABRICATION CHEMICALS. N = VELOCITY 5 MICROTUBULES

Chemicals		pH of the chemical	Number of Microtubules	Density of Microtubules
Control		6.9	20.0 ± 2.10	High
Removers/ Solvents	IPA	5.85	18.4 ± 2.93	High
	Acetone	7.71	14.8± 2.42	Intermediate
Developers	MIBK	6.16	18.6 ± 2.73	High
	TMAH	13.6	0.0	None
Resists	PMMA	—	16.0 ± 2.10	High
	UV5	—	13.2 ± 1.72	Intermediate

UV5 was baked at 135 °C for 1 min. The slides were then incubated with casein and kinesin using the standard procedure and washed in an acetone bath to strip the resists. Two flow cells were constructed using the slides and motility was checked. The protocol for the experiment is shown in Fig. 8. Motility was observed in regions where there was no resist, but no motility was detected in regions where there had been resist. The result of motility in the uncoated areas is shown in Table IV. Figs. 9 and 10 depict three screen shots showing the movement of microtubules on kinesin treated with acetone and acetone-dissolved PMMA, respectively. The velocity of microtubules under the influence of process chemicals was also examined and shown in Table V. No significant change in velocity was observed for treated kinesin relative to the control. These data indicate that resist removal does not affect kinesin motors, opening up the possibility of nanoscale patterning of motor proteins using conventional lithographic techniques.

D. Effect of Chemicals on Microtubules

To test the effect of process chemicals (acetone, IPA, TMAH, and MIBK) on microtubules, solutions of casein, kinesin, and microtubules were sequentially injected and incubated in flow cells, following the standard motility assay. After allowing time for the microtubules to bind and move over the bed of kinesin motors, developers and solvents were flowed into the flow cell. The experimental steps are shown in Fig. 11.

When this experiment was performed with the three process chemicals, no microtubules were observed on the surface. There could be several reasons for the above observations: 1) the chemicals attack rhodamine molecules in which case there will not be any fluorescence even in the presence of microtubules, or 2) microtubules do not depolymerize but are washed away during the chemical injection step, or 3) microtubules indeed depolymerize due to the chemicals. To rule out the possibility that the chemicals are quenching the rhodamine fluorescence, we imaged sur-

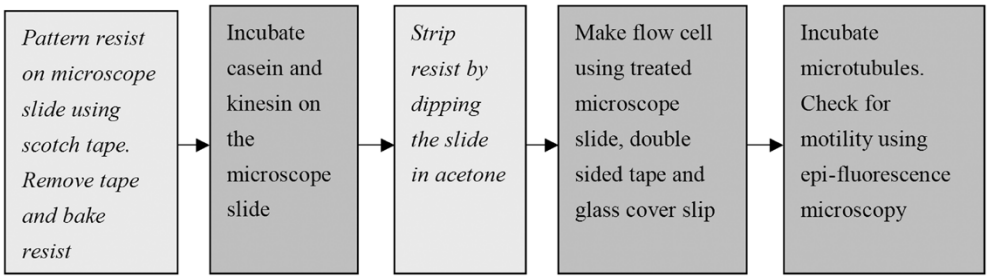

Fig. 8. Motility assay to test the effect of lithography resists on kinesin.

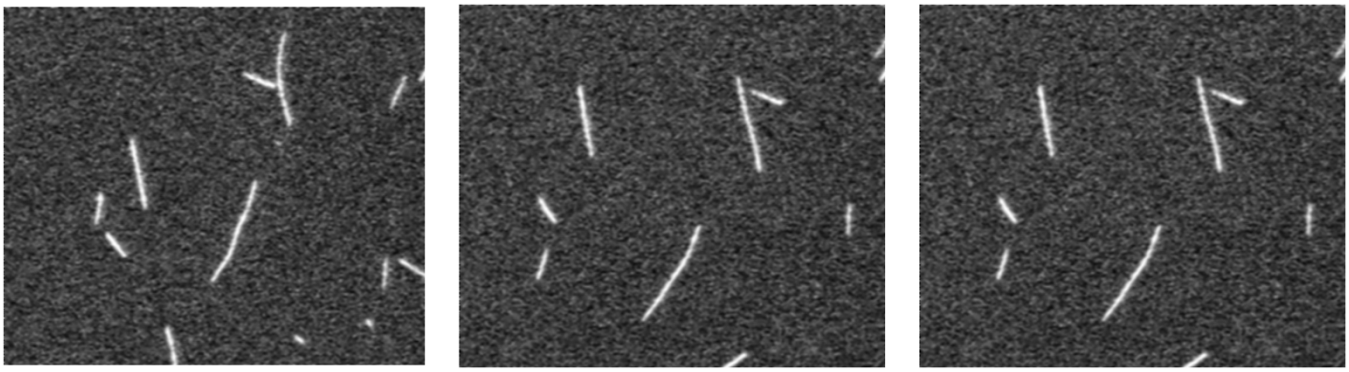

Fig. 9. Motility assay on acetone-treated kinesin ($t = 0$, 10, and 20 s).

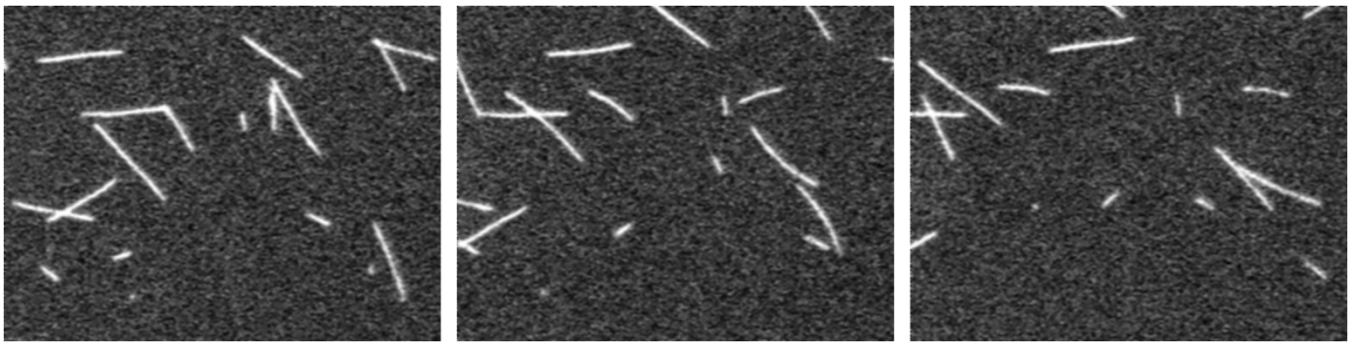

Fig. 10. Effect of PMMA removal on kinesin. Removed PMMA from the surface does not affect kinesin. Screen shots are taken at $t = 0$, 10, and 20 s.

TABLE V
RESULTS OF TREATMENT OF FABRICATION CHEMICALS ON MICROTUBULES. $N = 5$ SCREENS

Chemicals mixed with BRB80 in 1:4 ratio		pH of modified chemical	Motility	Density of Microtubules
Control		6.9	20.0 ± 2.10	High
Removers/ Solvents	Acetone	7.0	19.0 ± 2.45	High
	IPA	6.96	18.0 ± 4.51	High
Developers	MIBK	6.9	0.0	None
	TMAH	12.5	0.0	None

face-adsorbed rhodamine-labeled casein and found that the fluorescence remained in the presence of the chemicals. To rule out the possibility that the microtubules are simply being released from the surface and washed out, we mixed microtubules with equal volumes of the various chemicals, and visualized this solution under the microscope. When this experiment is performed in control buffer, numerous long microtubules are found, but in all four cases no microtubules were seen in the solutions. Hence, we conclude that pure acetone, IPA, TMAH, and MIBK all rapidly depolymerize microtubules.

One problem with exposing microtubules to these process chemicals is that they lack the buffering salts and counterions normally found in aqueous buffers. Hence, we diluted the chemicals in buffers to test whether microtubules could then survive exposure. Each of the process chemicals were mixed with four parts of BRB80 motility solution. This balanced the pH of acetone and IPA solutions to ~ 6.9–7.0, but did not bring down the pH of TMAH, while MIBK was immiscible with this aqueous buffer. The results of these experiments are shown in Table V. In the presence of the diluted acetone and IPA solutions, not only were microtubules stable, but kinesin motors were functional as well. On the other hand, microtubules were depolymerized in both TMAH and MIBK, presumably due to the high pH of TMAH and the nonpolar nature of MIBK.

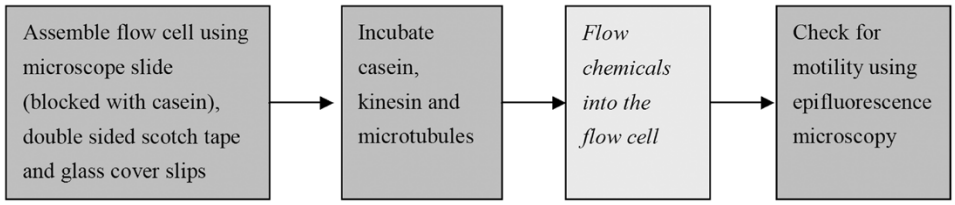

Fig. 11. Modified motility assay to test the effect of chemicals on microtubules.

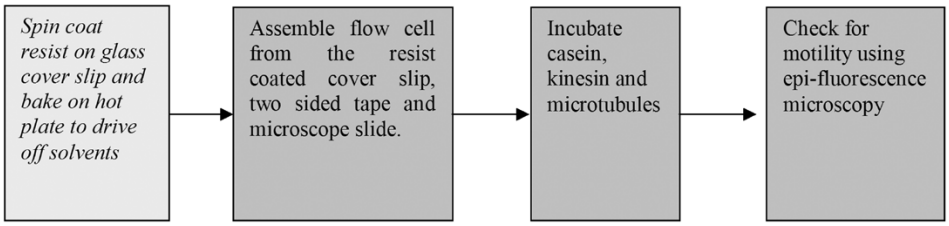

Fig. 12. Motility on lithography resists.

To better understand the interaction of solvents with these proteins, the function of motors and microtubules in modified acetone and IPA solutions was studied in greater detail. Ten microtubules were chosen randomly from each of the control, acetone, and IPA experiments after 40 min of exposure to the dilute solvent solutions and their lengths compared. Mean lengths $(\pm\text{SD})$ were 8.40 (± 2.49) μm, 7.98 (± 2.99) μm, and 5.20 (± 1.98) μm for control, acetone treated, and IPA treated microtubules, respectively. Hence, there appears to be no depolymerization in dilute acetone and very slow depolymerization in diluted IPA. To assess motor function, the microtubule gliding velocity was measured. The mean velocity of microtubules in modified acetone was 0.57 (± 0.01) μm/s $(N = 5)$ and in modified IPA was 0.39 (± 0.06) μm/s $(N = 5)$, compared with 0.80 (± 0.05) μm/s $(N = 5)$ in control buffer. In each case, all of the microtubules bound to the surface were found to be moving. Hence, 20% v/v acetone or IPA in motility buffer only reduces the motor speeds by a factor of two.

To determine whether a solution of 1:4 acetone:BRB80 still functions as a remover, PMMA was spin coated onto a glass slide at 3000 r/min for 40 s and baked for 90 s at 180 °C. The slide was then submerged into the 1:4 acetone: BRB80 solution. PMMA was stripped from the surface within 50 s. These studies indicate that process chemicals diluted with aqueous buffers can be used to remove conventional resists in the presence of kinesin motor proteins and microtubules, opening a new approach for patterning these proteins on surfaces at micrometer and submicrometer scales.

E. Motility on Lithography Resists

To determine whether kinesin motors are functional when adsorbed to UV5 and PMMA resists, motility assays were performed on coverslips that had been spin coated with these resists. Flow cells were made from the resist-coated cover slips and the standard solutions of kinesin and microtubules were sequentially introduced, as shown in Fig. 12. Motility was observed on both UV5 and PMMA (Table VI), demonstrating that features can be patterned in these resists, kinesins bound to the resists, and then microtubules transported on these features.

TABLE VI
MOTILITY RESULTS ON LITHOGRAPHY RESISTS. $N = 5$ FULL SCREENS

Resists	Moving Microtubules	Density of Microtubules
Control	20.0 ± 2.10	High
PMMA	18.6 ± 2.41	High
UV5	15.6 ± 1.85	High

F. Effect of UV Radiation on Kinesin

The final experiment tested whether kinesin is affected by UV light, which is routinely used to chemically modify resists for subsequent patterning. Casein and kinesin were incubated in a flow cell and UV light at an output power density of 12 mW/cm^2 produced from a mercury lamp was used to illuminate the samples for 1, 2, 5, and 10 min. Microtubules were flowed into the flow cell and motility was tested. We observed no change in microtubule density from the control experiment, indicating that UV light used in lithography does not affect kinesin motors. A flow chart summarizing the procedure used to test effects of UV light on kinesin is shown in Fig. 13.

Table VII summarizes the results of tests performed to assess the compatibility of lithography chemistries on casein, kinesin, and microtubules. A check $(\surd)$ indicates that experiments performed demonstrated that the proteins did not lose their functionality. A cross $(\times)$ indicates that the proteins did lose their functionality. In the case shown involving kinesin, it is believed that the protein was denatured by the high pH level. In the cases involving microtubules, it is believed that depolymerization has occurred.

IV. CONCLUSION

To obtain useful work from kinesin-microtubule mechanics, it is necessary to have the ability to pattern kinesin motors at desired locations and properly orient microtubules on surfaces and in channels. To assemble proteins in specific locations on a substrate surface, lithographic processes capable of directly interacting with kinesin motor proteins and microtubules are

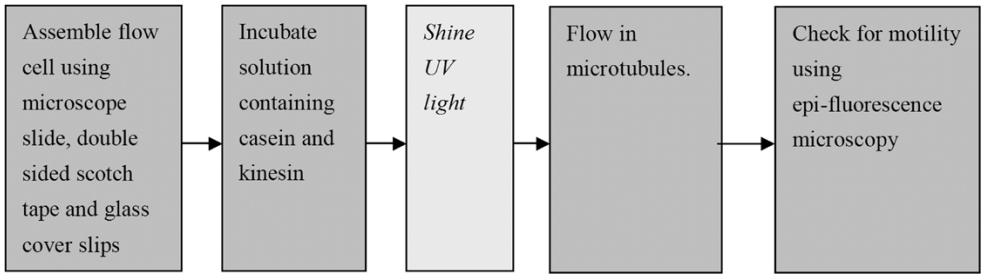

Fig. 13. Modified motility assay to test the effect of UV light on kinesin.

TABLE VII
SUMMARY OF COMPATIBILITY TEST OF PROCESS CHEMICALS WITH KINESIN SYSTEM

Chemicals		pH of the chemical	Casein	Kinesin	Microtubules
Removers/ Solvents	IPA	5.85	√	√	√*
	Acetone	7.71	√	√	√*
Developers	MIBK	6.16	√	√	×
	TMAH	13.6	√	×	×
Resists	PMMA	—	√	√	N/A
	UV5	—	√	√	N/A

*Removers and solvents were diluted five-fold in BRB80 buffer.

needed. A critical factor enabling such an approach is the compatibility of the biological molecules with micro- and nanofabrication processing chemicals. In this paper, we examined the effects of micro- and nanolithography chemistries on the blocking protein casein, the motor protein kinesin, and microtubules. It was found that casein can withstand high temperatures and almost all chemicals examined. Casein can, therefore, be adsorbed anytime during the lithography process without becoming denatured by temperature or by the chemistries used, allowing it to retain its functionality as a supporting structure for kinesin and as a means of preventing microtubules from binding to surfaces. Kinesin, with the exception of TMAH, can be used in conjunction with all the developers and solvents studied, opening new horizons in the field of protein patterning. Kinesin motor proteins can be patterning on substrates using nanolithography without losing their functionality. Microtubules were by far the most susceptible to the chemistries used in fabrication. However, if the resist developers were diluted in aqueous buffers, they still worked as strippers, and they no longer depolymerized microtubules. Hence, in addition to patterning kinesin motors, microtubules can also be potentially patterned on surfaces with these techniques.

One application of our studies is the patterning of motor proteins on substrates via direct e-beam lithography without the use of any linker or self-assembly technique in particular the patterning of kinesin using PMMA and its respective developer (MIBK) and stripper (acetone). Additional studies are required to understand the function of the casein present in kinesin solution during the incubation step. Studies performed suggest that casein and kinesin are engaging in a dynamic coassembly process to form an active layer of functional motor proteins on the surface of the glass slide. Furthermore, techniques to pattern multiple proteins need to be developed and the interaction between proteins after patterning needs to be studied. It is also essential to orient and position microtubules to realize hybrid devices. One of the largest obstacles precluding the patterning of motor proteins and the precise placement and orientation of microtubules is their instability toward micro- and nanofabrication processes. This paper is a step toward the goal of fabricating hybrid devices, addressing the important missing link of biocompatibility of micro- and nanofabrication processes with biological proteins.

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Vivek Verma was born in Lucknow, India. He received the engineers degree in metallurgy and materials in 2002 from the Indian Institute of Technology, Madras.

His research interests are in protein patterning, nanoscale fabrication, and synthesis of hybrid synthetic and biological devices. He is currently working on molecular motor-powered devices in the Department of Engineering Science and Mechanics, Pennsylvania State University, University Park. His work includes developing biologically compatible nanofabrication processes and fabricating synthetic devices powered by biological motors.

W. O. Hancock received the B.S. degree in biomedical engineering and electrical engineering from Duke University, Durham, NC, the Ph.D. in bioengineering from the University of Washington, Seattle, and received postdoctoral training in the Department of Physiology and Biophysics, University of Washington.

He is an Assistant Professor of Bioengineering at Pennsylvania State University (Penn State), University Park, and Motors Thrust Leader in the Penn State Center for Nanoscals Science, a Materials Research Science and Engineering Center funded by the National Science Foundation. His research interests are on both fundamental investigations on kinesin motor proteins and the microtubule cytoskeleton as well as applied investigations aimed at incorporating the biological motors into microfabricated systems.

Jeffrey M. Catchmark received the B.S. degree in electrical engineering from Pennsylvania State University, University Park, in 1990 and the Ph.D. degree in electrical engineering from Lehigh University, Bethlehem, PA, in 1995.

He is the Operations Manager of the Pennsylvania State University Nanofabrication Facility and co-Director of the Pennsylvania State University site of the National Science Foundation National Nanotechnology Infrastructure Network (NSF NNIN). He is also an Assistant Professor of engineering science and mechanics in the College of Engineering. He has worked as a Consulting Member of the Technical Staff at AT&T Bell laboratories, a Member of the Technical Staff at Princeton Electronic Systems, Inc., and Chief Optoelectronics Engineer at C-COR Electronics, Inc. His research interests include biomimetics, bionanotechnology, molecular nanofabrication, and photonics.