

## Microtubule Motors in Microfluidics

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### Abstract

Kinesin motor proteins carry out a range of transport functions in eukaryotic cells including long-distance transport in neurons and the movement of chromosomes during cell division. Because emerging microfluidic devices utilize channel geometries similar to cellular scales and because these devices generally require transport through these channels, there is significant interest in incorporating biomotor-driven transport into microfluidic devices. Kinesin-driven transport has the advantage of being able to work against concentration gradients and bulk fluid flow, and microtubules can be functionalized to carry a range of cargo. This chapter describes the foundational methods for generating kinesin-driven transport in engineered microchannels. Protocols are described for expressing and purifying recombinant kinesin, purifying and fluorescently labeling bovine brain tubulin, and carrying out the microtubule gliding assay. General design considerations are presented for integrating kinesins and microtubules into microchannels, followed by a detailed description of a microfluidic device prototype that successfully integrates microtubule-based transport.

### Key terms

kinesin  
microtubule  
transport  
motor protein  
bionanotechnology

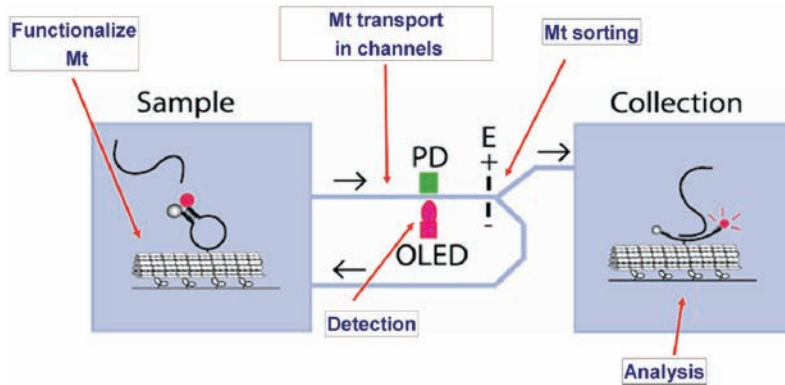
## 13.1 Introduction

In emerging lab-on-a-chip technologies, the microfluidic devices designed to shrink analytical tests and biosensors down to microscale dimensions generally require some manner of mechanical actuation. While these mechanical forces can be achieved by pressure-driven fluid pumps, electrically or magnetically driven movement, electroosmotic flow, or other approaches, there is a continuing need in this area for reliable mechanical actuation and directed transport. In eukaryotic cells, kinesin motor proteins transport intracellular cargo and provide the mechanical forces underlying chromosome movements and other mechanical processes. These motor proteins utilize the chemical energy of ATP hydrolysis to walk on protein filaments, called microtubules. This biologically driven movement can be reconstituted under a microscope by immobilizing kinesin motors on a substrate (such as a glass coverslip) and observing the movement of microtubules over the surface. Building on the robust microscale transport in this “microtubule gliding assay,” there has been considerable interest in utilizing kinesin-driven microtubule motion for nano/microscale transport applications in lab-on-a-chip devices [1–3]. While the development of lab-on-a-chip devices is primarily based on microfluidics technology, integration of kinesin-driven motion in microfluidic devices has several advantages. First, motors can transport cargo against concentration gradients. Second, no bulk fluid flow or pumping mechanisms are required, easing the fabrication and power requirements of such devices. Third, microtubules and kinesins can be modified to bind specific cargo, enabling their use in biosensing and separation processes.

A schematic of a potential device of this type is illustrated in Figure 13.1. This device is designed to detect viral RNA in biosensing applications, or to detect mRNA from a cell lysate, which would enable gene expression profiling, in principle down to single cell levels [1]. In the design, microtubules functionalized with single-stranded DNA constructs (molecular beacons) are transported through microchannels functionalized with kinesin motors [4]. These mobile probes capture and transport specific RNA and report this sequence-specific binding by unquenching their fluorescence. Integrated optical sensors and electrodes detect and guide the microtubules such that the cargo is separated from the heterogeneous sample and is concentrated in the collection chamber. If successful, this device could have a range of uses, and it is only one of many biomotor-driven devices that have been envisioned. However, successful integration of microtubules in such lab-on-a-chip devices requires (1) the ability to achieve directional motion of microtubules in enclosed microfluidic channels, (2) the ability to bind and transport cargo, and (3) the ability to detect and sort microtubules based on the presence of cargo. This is an active area of research and key blocks of technology have been developed by our group and others. In this chapter we detail the experimental methods involved in purifying kinesin motors and microtubules, techniques for modifying these proteins for use in microfluidic channels, and approaches for optimizing the design of microfluidic channels to achieve robust kinesin-driven transport.

## 13.2 Materials

Relevant materials, reagents, and equipment are listed below. Both the “Materials” and the “Methods” sections are divided into four subsections: (1) Kinesin expression and



**Figure 13.1** Schematic for biosensor/bioseparation device-based kinesin-driven transport. Microtubules are functionalized with molecular beacons that bind RNA and fluoresce upon binding. The beacon-functionalized microtubules are transported through microfluidic channels to which kinesin motor proteins are adsorbed. An integrated photodiode (PD) and organic light emitting diode (OLED) detect the presence of RNA cargo on the microtubule and electrodes (E) direct the microtubules at a bifurcation, directing cargo-loaded microtubules into the sample chamber for further analysis such as PCR amplification or sequencing. (Image taken, with kind permission from Springer Science+Business Media, from Jia et al. [1].)

purification, (2) tubulin purification and labeling, (3) microtubule gliding assay, and (4) integrating motility into microchannels. Unless specified, the reagents mentioned below can be obtained from alternative vendors, and for reagents where no source is specified, Sigma, Fischer, or any equivalent source are sufficient.

### 13.2.1 Kinesin expression and purification materials

#### 13.2.1.1 Kinesin reagents

- Kinesin expression plasmid in BL21(DE3) bacterial cells
- LB media (Qbiogene, Cat # 3002-092)
- Ampicillin sodium salt (Sigma-Aldrich, Cat # A9518)
- Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, Cat # I5502)
- Phenylmethanesulfonyl fluoride (PMSF) (Sigma-Aldrich, Cat # P7626)
- Adenosine-5'-triphosphate (ATP) (Plenum Scientific Research, Inc., Cat # 0163-04)
- $\beta$ -Mercaptoethanol ( $\beta$ ME) (Sigma-Aldrich, Cat # M3148)
- Dithiothreitol (DTT) (Bio-Rad, Cat # 161-0611)
- Sodium phosphate monobasic (Sigma-Aldrich, Cat # S3139)
- Sodium phosphate dibasic (Sigma-Aldrich, Cat # S3264)
- Sodium chloride (Sigma-Aldrich, Cat # S3014)
- Magnesium chloride (Sigma-Aldrich, Cat # 68475)
- Sodium hydroxide (NaOH)
- Sucrose (Sigma-Aldrich, Cat # S0389)
- Imidazole (Sigma-Aldrich, Cat # I5513)

### 13.2.1.2 Kinesin buffers

- Lysis buffer: 50-mM sodium phosphate, 300-mM NaCl, 40-mM imidazole, 5-mM  $\beta$ ME, pH 8.0.
- Wash buffer: 50-mM sodium phosphate, 300-mM NaCl, 60-mM imidazole, 100- $\mu$ M MgATP, 5-mM  $\beta$ ME, pH 7.0.
- Elution buffer: 50-mM sodium phosphate, 300-mM NaCl, 500-mM imidazole, 100- $\mu$ M MgATP, 5-mM  $\beta$ ME, 10% (w/v) sucrose, pH 7.0.

*Note:* For making these phosphate buffers, either combine monobasic and dibasic forms to approximate the final pH and then adjust accordingly, or use monobasic form and pH with concentrated NaOH. pH buffers before adding  $\beta$ ME and sucrose.  $\beta$ ME has a lifetime of days in these buffers, so prepare accordingly: refrigerate sucrose containing solutions to prevent bacterial growth. One convenient approach is to make up buffers without ATP,  $\beta$ ME, or sucrose, and add these just before using.

### 13.2.1.3 Kinesin equipment

- 2L Erlenmeyer flask (preferably baffled)
- Temperature-controlled incubator with orbital shaker
- Sonicator for lysing bacterial cells (Branson Ultrasonics)
- AKTA-FPLC chromatography system (or equivalent)
- Ni-NTA agarose chromatography column (5 ml)
- SDS-PAGE gel electrophoresis equipment

## 13.2.2 Tubulin purification materials

### 13.2.2.1 Tubulin reagents

- Cow brains from freshly slaughtered cows (obtain from local slaughterhouse)
- Leupeptin hydrochloride (Sigma-Aldrich, Cat # L9783)
- Trypsin inhibitor (Sigma-Aldrich, Cat # T9253)
- Pepstatin A (Sigma-Aldrich, Cat # P5318)
- Aprotinin (Sigma-Aldrich, Cat # A6103)
- $N_\alpha$ -*p*-Tosyl-L-arginine methyl ester hydrochloride (TAME) (Sigma-Aldrich, Cat # T4626)
- Tosyl-L-phenylalanyl-chloromethane (TCPK) (Sigma-Aldrich, Cat # T4376)
- Ethylene glycol-bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (Sigma-Aldrich, Cat # E3889)
- Ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, Cat # E9884)
- L-Glutamic acid monosodium salt monohydrate (sodium glutamate) (Sigma-Aldrich, Cat # G2834)
- L-Glutamic acid potassium salt monohydrate (potassium glutamate) (Sigma-Aldrich, Cat # G1501)
- Piperazine-*N,N'*-bis(2-ethanesulfonic acid) (PIPES) (Sigma-Aldrich, Cat # P6757)

- N-(2-Hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) (HEPES) (Sigma-Aldrich, Cat # H3375)
- Glycerol (Sigma-Aldrich, Cat # G5516)
- DMSO anhydrous (Sigma Aldrich, Cat # 276855)
- 2'-Guanosine 5'-triphosphate, sodium salt (GTP) (Jena Bioscience, Cat # NU-1012)
- Paclitaxel (Taxol) (Sigma Aldrich, Cat # T7402) (*Note:* Required for stabilizing microtubules. Dissolve at 1 mM in DMSO for use and store frozen aliquots. Taxol inhibits cell division and DMSO aids absorption into skin, so avoid contact with skin.)
- 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (Rhodamine-NHS) (Invitrogen, Cat # C1171)

### 13.2.2.2 Tubulin buffers and solutions

- PBS buffer—20-mM sodium phosphate, 150-mM NaCl, pH 7.4
- Glutamate buffer—1.0M sodium glutamate, 2-mM EGTA, 0.1-mM EDTA, 2-mM MgCl<sub>2</sub>, pH 6.6
- BRB80 buffer—80-mM K-PIPES, 1-mM EGTA, 1-mM MgCl<sub>2</sub>, pH 6.85. (Can make with mixture of PIPES acid and salt forms or use all acid form and pH with KOH. PIPES is only minimally soluble below pH 6 and will dissolve as base is added.)
- 5x BRB80 buffer—400-mM K-PIPES, 5-mM EGTA, 5-mM MgCl<sub>2</sub>, pH 6.85
- HEPES/40% glycerol (labeling buffer)—0.1 M HEPES, 1-mM MgCl<sub>2</sub>, 1-mM EGTA, 40% glycerol (v/v), pH 8.6
- Quench solution—0.5 M potassium glutamate, pH 8.6
- Depolymerization solution—50-mM potassium glutamate, 0.5-mM MgCl<sub>2</sub>, pH 7.0

### 13.2.2.3 Tubulin equipment

- Waring blender
- Temperature-controlled 37°C water bath
- Beckman Ultracentrifuge, Type 19 and 50.2 Ti rotors, and centrifuge tubes/bottles
- Tissue tearor
- Beckman Airfuge
- UV-Vis Spectrophotometer

## 13.2.3 Microtubule gliding assay materials

- Fisher's Finest plain glass microscope slides (Fisher Scientific, Cat # 12-544-1)
- Corning 18 x18-mm coverslips (Fisher Scientific, Cat # 12-519A) (*Note:* Corning coverglass works best for gliding assay compared to other coverglasses. Different sizes can be used)
- Double-stick tape
- Casein from bovine milk (Sigma-Aldrich, Cat # C3400) (*Note:* This is an important reagent that is used to optimize surfaces for functional kinesin adsorption. Casein should be dissolved in BRB80 at ~20 mg/ml and stored at -20°C. Dissolve 2.5g

casein in 25-ml BRB80 buffer in 50-ml Falcon tube and rock for 4 hours to overnight to dissolve (keep at 4°C if > 4 hours). Next, spin in centrifuge (10 minutes at >100,000 x g is good, but less is acceptable) to pellet insoluble material. Finally, filter using 0.2- $\mu$ m syringe filters. You will need to go through a number of filters as they tend to clog. Assess concentration by UV absorbance (1 mg/ml = 1  $A_{280}$ ), if > 20 mg/ml, then dilute, aliquot, and store at -20°C.)

- D-glucose (Sigma-Aldrich, Cat # G7528)
- Glucose oxidase (Sigma-Aldrich, Cat # G2133)
- Catalase (Sigma-Aldrich, Cat # C515)

*Note:* Glucose, glucose oxidase, and catalase are parts of an antifade oxygen scavenging system. It is convenient to make up stock solutions in BRB80 at 2M, 2 mg/ml, and 0.8 mg/ml, respectively, store 10- $\mu$ l aliquots at -20°C, and thaw and dilute 100-fold into motility solution for motility experiments.

- Fluorescence microscope with 60x or 100x objective.

*Note:* Generally a CCD camera, monitor, and recording device such as a VCR or computer is necessary for optimal visualization and analysis. While expensive enhanced CCD cameras such as the Roper Cascade 512 work well, we have also had success with a Genwac 902H CCD camera, which can be found online for less than \$400.)

### 13.2.4 Microfabrication materials

#### 13.2.4.1 Microfabrication reagents and supplies

- Borosilicate glass substrates (1-mm thick)
- ProSciTech 50 × 50-mm coverslips
- 996-kDa poly(methyl methacrylate) (PMMA)
- Chlorobenzene
- Acetone
- Isopropyl alcohol (IPA)
- Sulfuric acid ( $H_2SO_4$ )
- Hydrogen peroxide ( $H_2O_2$ )
- Piranha solution ( $H_2SO_4:H_2O_2 = 4:1$ )
- 1811 photoresist
- Tetramethylammonium hydroxide (TMAH) developer
- SU-8 photoresist
- SU-8 developer
- Chrome and gold for deposition
- Gold for electroplating
- Chrome etch solution
- Gold etch solution
- Hydrogen fluoride (HF)
- Ammonium fluoride ( $NH_4F$ )
- Buffered oxide etch (BOE) ( $NH_4F/HF 10:1$ )

*Note:* Piranha solution sulfuric acid, hydrogen fluoride, ammonium fluoride, Cr and Au etch solutions and BOE are extremely corrosive and precautions should be taken to prevent any contact with skin or clothing. Always use proper safety procedures when using these chemicals.

#### 13.2.4.2 Microfabrication equipment

- Karl Suss MA-55 aligner
- Photoresist spinner
- Hotplate
- Chemical wet bench
- DC power supply (for Au electroplating)
- Resistive filament thermal evaporator
- RIE equipment (for oxygen plasma)
- Warner 100 hydraulic press laminator

## 13.3 Methods

### 13.3.1 Kinesin expression and purification

A number of kinesins have been investigated from many different organisms, but most of the biophysical investigations to date and virtually all of the microfluidics research using kinesins have employed conventional kinesin (also called Kinesin-1 [5]). Our investigations have employed full-length *Drosophila* conventional kinesin that contains a hexaHis-tag on its C-terminus for purification [6, 7]. The full-length gene can be truncated to remove the tail domain and the tail replaced with a biotinylation sequence, green fluorescent protein, or other sequences. In our work we also employ a headless kinesin construct that retains the rod and tail domains and hexaHis tag, but lacks its motor domain [6, 8]. Below, we provide a detailed protocol for expressing this kinesin in bacteria, purification by Ni column chromatography, and freezing for long term storage.

This protocol is for full-length *Drosophila* conventional kinesin in a pET plasmid expressed in BL21(DE3) *E. coli* cells, though it will work for other hexaHis-tagged kinesins. It generally follows protocols described in Hancock et al. [6] and in Coy et al. [7]. Related protocols can be found in a book of kinesin protocols [9] and on the Kinesin home page (<http://www.cellbio.duke.edu/kinesin/>).

The amino acid sequence of the protein and the nucleotide sequence of the entire plasmid (pPK113) are online (entry AF053733 at <http://www.ncbi.nlm.nih.gov/pubmed/>). Furthermore, this plasmid is available to academic researchers from the plasmid depository Addgene (<http://www.addgene.org>). The method for preparing and purifying these proteins is described below:

1. Prepare LB media and autoclave to sterilize. After the media cools to room temperature, add 50 mg of ampicillin sodium salt (final concentration 100 mg/L).
2. Pipette 5 ml of LB+Amp into a 15-ml Falcon tube and inoculate it with a colony of plasmid-containing BL21(DE3) cells. Incubate overnight (16 hours) at 37°C while shaking at 250 rpm.

3. Transfer the 5-ml overnight culture to 500-ml LB medium in a 2L Erlenmeyer flask and incubate at 37°C while shaking at 250 rpm until the culture grows to an optical density at 600 nm of 0.5 to 1.0. This typically requires 3 to 6 hours of growth.
4. Cool the culture to room temperature on ice and add 45 mg of IPTG (final conc. 0.5 mM) to induce protein expression. Incubate at 20°C for 3 hours while shaking at 250 rpm to express protein. (*Note:* Expression at this temperature improves solubility of the expressed protein.)
5. Transfer culture to centrifuge bottles and harvest cells by centrifuging for 10 minutes at 6,000 × g. Resuspend pellet in 25 ml of lysis buffer. If desired, cells can be flash-frozen in liquid nitrogen and stored at -80°C at this point for later purification.
6. Thaw cells in 37°C water bath if necessary. Add 0.1-mM ATP and 1-mM PMSF to the solution and lyse the cells by sonicating the cells on ice. Use 4 to 6 pulses of 20 seconds each on duty cycle 5 to completely lyse the cells. Cell lysis can also be carried out by French press [6]. (*Note:* one indicator of lysis is that the solution goes from cloudy to clear due to fragmenting the light scattering bacterial cells. Another test is to run a gel of the pellet and supernatant from step 7—incomplete lysis results in a large portion of the expressed protein in the pellet.)
7. To remove cell debris and insoluble components, centrifuge the cell lysate at 100,000 × g for 30 minutes at 4°C. The supernatant (clarified lysate) contains the soluble recombinant protein along with bacterial proteins.
8. While the cell lysate is spinning, equilibrate the Ni-NTA column by flowing through 10x column volumes (50 ml) of lysis buffer at a flow rate of 5 ml/min.
9. Apply clarified lysate to the column at a flow rate of 2 ml/min to bind hexaHis-tagged protein to the column. After loading, wash column with 10x column volume (50 ml) wash buffer at a flow rate of 5 ml/min to remove unwanted bacterial proteins. The UV absorbance readout on the chromatography system (280 nm) should fall from a peak during loading to a flat baseline at the end of the wash. (*Note:* if greater purity of final eluted protein is desired, imidazole in wash buffer can be raised to 80 or 100 mM.)
10. Elute the kinesin by flowing elution buffer over the column at a flow rate of 1 ml/min. Collect the elution in 0.5-ml fractions. Monitor elution by UV absorbance—because imidazole has an absorbance at 280 nm, there will be an absorbance jump due to the high imidazole concentration in the elution buffer, but a peak or at least a shoulder from the eluted protein should be observable before the absorbance plateaus. The fractions corresponding to the peak absorbance will contain the purified protein. The peak fractions can also be determined and the protein concentration quantified by a UV-Vis spectrophotometer or by running an SDS-PAGE gel of the elution fractions. Typical yields are 1 to 2 ml at 100 µg/ml concentration for full-length kinesin, though they can be significantly higher for truncated constructs. (*Note:* microtubule gliding assays using 5x-diluted elution fractions can also be run to identify the peak fractions.)
11. The purified protein can be flash-frozen in liquid nitrogen and stored at -80°C. (*Note:* the 10 % sucrose in the elution buffer acts as a cryoprotectant.)



*Notes:* Kinesin frozen at  $-80^{\circ}\text{C}$  is stable for years, but samples stored at  $4^{\circ}\text{C}$  only last for days and samples at room temperatures lose activity over hours. Low-concentration samples can be stabilized somewhat by adding 0.5-mg/ml casein or BSA—part of the activity loss in samples with low kinesin concentrations is due protein adsorption to the sides of the tubes, which is minimized by adding these inert proteins.

### 13.3.2 Tubulin purification and labeling

Microtubules, which are 25 nm in diameter and can be tens of microns in length, are made up of  $\alpha$ - $\beta$  heterodimers of the protein tubulin [10]. There are no established techniques for bacterial expression of tubulin, so the standard approach is to purify tubulin from cow or pig brains. Microtubules are then polymerized from this purified tubulin and stabilized against depolymerization using the drug Taxol. There are published protocols for large scale tubulin purification, including those by Williams and Lee [11] and Castoldi and Popov [12], as well as online protocols from the Salmon Lab (<http://www.bio.unc.edu/Faculty/Salmon/lab/protocolsporcinetubulin.html>) and Mitchison Lab (<http://mitchison.med.harvard.edu/protocols/tubprep.html>) Web sites. Here, we describe an adapted protocol for isolating bovine brain tubulin that has worked reproducibly in our laboratory. The procedure involves polymerizing microtubules, pelleting them by centrifugation to remove unwanted soluble proteins, and then depolymerizing and centrifuging to remove unwanted insoluble proteins. The key hurdle in isolating pure tubulin is removing unwanted microtubule associated proteins (MAPs). Earlier protocols used a final phosphocellulose cation exchange column step to remove these positively charged MAPs from the negatively charged tubulin, while this protocol uses high concentrations of glutamate in the buffers to reduce the affinity of the MAPs for the microtubules.

Note that tubulin is commercially available from Cytoskeleton, Inc. ([www.cytoskeleton.com](http://www.cytoskeleton.com)). For small-scale studies, this is a reasonable source and is cost-effective. However, for full control over the materials, to enable the ability to carry out diverse modifications of the protein, and for applications where the large material requirements make purchasing it prohibitively expensive, it is desirable to purify the tubulin from the source.

#### 13.3.2.1 Protocol for tubulin purification

The procedure starts with harvesting cow brains, homogenizing them to break open the cells, and clarifying the homogenate by centrifugation to obtain a solution of soluble intracellular proteins. This is followed by two cycles of polymerization/centrifugation/depolymerization/centrifugation to purify tubulin away from unwanted cellular proteins and MAPs. When carried out successfully, the protocol yields roughly 500 mg of pure tubulin at  $>99\%$  purity, though yields will vary.

1. Prepare 2L each of PBS buffer and glutamate buffer and chill them to  $4^{\circ}\text{C}$ .
2. Obtain two cow brains from freshly slaughtered cows. Transport the brains submerged in ice-cold PBS buffer in an ice-filled cooler. (*Note:* It is critical to process the brains within  $\sim 1$  hour of slaughter. Delays in processing reduce yield due to degradation of tubulin.)

3. Dissect the brains on ice, discarding brain stem, cerebellum, and corpus collosum. Strip meninges (tough filaments on outside of brain) and remove blood clots, then rinse dissected cerebrums with ice-cold PBS. Work quickly and try to balance speed with thoroughness of cleaning—90% is a good rule here.
4. Prepare protease inhibitor cocktail by dissolving the protease inhibitors listed in Table 13.1 in 1 ml of their respective solvents.
5. Weigh cerebrums (in preweighed beaker) and transfer to a chilled Waring blender. Add 50% v/w of chilled glutamate buffer (for example: add 350 ml for 700g of brains). Add protease cocktails and 1-mM ATP, 0.25-mM GTP, 4- $\mu$ M DTT, 0.1%  $\beta$ ME.
6. Homogenize the brains (blending in 4 $\times$ 10-second high-speed pulses, with 15-second pauses between pulses to minimize heating), and distribute the homogenate into 250 ml centrifuge bottles.
7. Spin the homogenate at 50,000  $\times$  g (i.e., Beckman type 19 rotor, 19,000 rpm [53,900  $\times$  g,  $k = 951$ ]) for 60 minutes at 4°C to clarify the solution. Pour the supernatants gently into a 1L graduated cylinder without disturbing the soft pellets. Discard pellets.
8. To polymerize tubulin add prewarmed (37°C) glycerol to the supernatant to a final concentration of 33% (v/v) glycerol, and add 0.5-mM GTP, 1.5-mM ATP each dissolved in ~1 ml of buffer. Mix well by inverting (cover with Parafilm and hold in place with your hand) and transfer the contents to a 500-ml stainless steel beaker. Incubate Parafilm-covered beaker in 37°C water bath for 1 hour to polymerize microtubules. (*Note:* Solution should become more viscous and take on a subtle opalescence if polymerization is proceeding.)
9. In the meantime, warm the centrifuge and rotor to 37°C. After polymerization, transfer microtubule solution to prewarmed centrifuge tubes and centrifuge at 37°C at 300,000  $\times$  g for 30 minutes to pellet microtubules. We used a Beckman 50.2 Ti rotor at 50,000 rpm [302,000  $\times$  g,  $k = 133$ ]).
10. Discard supernatant containing unwanted proteins, weigh the pellets, and resuspend pellets in ice-cold glutamate buffer. Add 1 ml of cold buffer to each centrifuge tube and physically dislodge the pellet from the tube using a spatula. Pool the pellets in a 250-mL glass beaker on ice and add ice-cold buffer. Choose volume for resuspension to be roughly 1/3 of polymerization volume to maintain high tubulin concentrations. To depolymerize microtubules, homogenize pellets with tissue tearor and incubate 30 minutes on ice with additional tissue tearor homogenization during the incubation.

**Table 13.1** Protease Inhibitors

Component	Final	Solvent
Leupeptin	5 mg/mL	DI water
Trypsin inhibitor	10 mg/mL	DI water
Pepstatin	1 mg/mL	DMSO
Aprotinin	1 mg/mL	DMSO
TAME	1 mg/mL	DMSO
TPCK	1 mg/mL	DMSO
PMSF	100 mM	Ethanol

11. In the meantime, chill the rotor and centrifuge to 4°C. Following incubation, clarify the depolymerized tubulin by centrifuging at 300,000 × g for 30 minutes at 4°C. Save supernatant and discard pellets.
12. Repeat tubulin cycling steps 8 to 11, with the exception that the pellets are resuspended in cold BRB80 buffer instead of glutamate buffer in step 10. This “twice-cycled tubulin,” cycled in high (glutamate) buffer to remove microtubule associated proteins, is equivalent to “PC tubulin” obtained by passing the solution over a phosphocellulose column.
13. The tubulin concentration in the final supernatant can be quantified by UV absorbance at 280 nm ( $1 A_{280} = 1 \text{ mg/ml tubulin}$ ). Aliquot tubulin into cryotubes, flash-freeze on liquid nitrogen, and store at -80°C. A helpful rule to remember is that 1 mg/ml of the 100-kD tubulin dimer equals 10- $\mu$ M tubulin.

### 13.3.2.2 Protocol for labeling tubulin with rhodamine

The following protocol describes labeling of pure tubulin (obtained in the previous step) by rhodamine succinimidyl ester (rhodamine-NHS), a reactive form of the rhodamine fluorophore. There are a range of fluorophores that can be purchased in this reactive form as well as biotin and other compounds. These can be purchased from Molecular Probes/Invitrogen or other sources, and this protocol is suitable for microtubule labeling using these compounds as well. This protocol is adapted from a widely used protocol in the literature [13].

1. Warm the rotor (i.e., Beckman 50.2 Ti) and centrifuge to 37°C. Thaw a 100-mg aliquot of purified tubulin at 37°C (can be scaled up or down). Add prewarmed glycerol to 33% final v/v, along with 1-mM GTP and 5-mM MgCl<sub>2</sub> and polymerize for 30 minutes in a 37°C water bath.
2. Spin the polymerized solution at 300,000 x g for 30 minutes to pellet microtubules. Discard the supernatant and resuspend the pellet in 3.5-ml prewarmed HEPES/40% glycerol (with 1-mM GTP and 5-mM MgCl<sub>2</sub>), and maintain the solution at 37°C. (*Note:* Make sure the microtubules are well suspended by pipetting up and down repeatedly.) Quickly measure the tubulin concentration using UV absorbance.
3. Just prior to labeling, dissolve the rhodamine-NHS dye (5-(and-6)- carboxytetramethylrhodamine, succinimidyl ester, Invitrogen) in DMSO to a final concentration of 50 mM. Based on the microtubule concentration add the dye in the ratio of 20 dye molecules per tubulin heterodimer and incubate at 37°C for 15 minutes. (*Notes:* (1) It is advisable to briefly centrifuge the dissolved dye solution (i.e., 1 minute in any bench top centrifuge) to remove any undissolved dye crystals that will later pellet with the microtubules. (2) HEPES buffer at pH 8.6 is used because NHS reactive groups label ionized (NH<sub>3</sub><sup>+</sup>) amino groups and this is closer to the pKa for the lysine side chains that are labeled. We have, however, had success with labeling in BRB80 at pH 6.8, so this is an option for optimizing labeling.)
4. Stop reaction by adding K-glutamate to a final concentration of 50 mM. Centrifuge the solution at 300,000 x g for 30 minutes at 37°C to pellet microtubules away from unincorporated dye.
5. Discard supernatant and resuspend the pellet in 2 ml of cold depolymerization buffer. Incubate on ice for 30 minutes, with occasional gentle vortexing to depolymerize.

6. Centrifuge the solution in a Beckman Airfuge at 30 psi for 10 minutes to remove insoluble material and protein aggregates. Remove the supernatant and add 20% volume of 5x BRB80 buffer.
7. Polymerize microtubules by adding 1-mM GTP, 5-mM MgCl<sub>2</sub>, and 5% DMSO and incubating at 37°C for 30 minutes. Centrifuge the solution 5 minutes in the Airfuge, remove the supernatant, resuspend the microtubules in 1.5 ml of cold BRB80, and depolymerize on ice for 30 minutes.
8. Centrifuge the solution one last time in the Airfuge and harvest supernatant containing the labeled tubulin. To quantify the yield and the dye:tubulin ratio, measure the absorbance in a UV/Vis spectrophotometer at 280 and 555 nm. The dye has absorbance at both 280 and 555 nm (absorption coefficient  $\epsilon_{555} = 49,291 \text{ M}^{-1}\text{cm}^{-1}$  and  $\epsilon_{280}/\epsilon_{555} = 0.2476$ ) and tubulin has an absorbance at 280 nm ( $\epsilon_{280} = 101,900 \text{ M}^{-1}\text{cm}^{-1}$ ). The dye concentration is obtained at 555 nm, and the tubulin concentration is obtained at 280 nm after subtracting out the dye absorbance at this wavelength. A 1:1 dye:tubulin ratio is a good target, though even minimally labeled tubulin is usually useful. Aliquot the labeled tubulin, flash-freeze in liquid nitrogen, and store at -80°C.

### 13.3.2.3 Cycling tubulin and mixing labeled unlabeled tubulin for optimal functionality

For visualizing microtubules by epifluorescence microscopy, we have found that the optimum dye:tubulin ratio is 1:5. This ratio gives microtubules that are bright enough to visualize, and the dye has no apparent effect on microtubule or kinesin function. The approach is to combine labeled and unlabeled tubulin at specified ratios and to aliquot, freeze and store this tubulin for use in motility experiments. We have observed that microtubules containing 1:1 dye:tubulin ratios have different polymerization characteristics, so caution should be used when working with highly labeled microtubules, but the optimum dye:tubulin ratio may vary for different applications, and can be modified. Furthermore, multifunctional microtubules that contain tubulin labeled with combinations of fluorophores or with biotin can be assembled (i.e., biotinylated, rhodamine-labeled microtubules). When making up tubulin solutions, unlabeled tubulin is typically cycled, quantified, and then combined with labeled tubulin. The following cycling protocol ensures that all of the tubulin is active and removes any dead protein.

1. Thaw a 50-mg aliquot of tubulin in a 37°C water bath. Add 1-mM GTP, 5-mM MgCl<sub>2</sub>, and 5% DMSO and incubate at 37°C for 30 minutes to polymerize.
2. Centrifuge the polymerized microtubules at 300,000 × g for 30 minutes (i.e., 50,000 rpm in a 50.2Ti rotor) at 37°C. Discard supernatant, resuspend the pellet in 1.5 ml of cold BRB80 buffer, and depolymerize on ice for 30 minutes.
3. Centrifuge the solution in an Airfuge for 10 minutes and harvest the supernatant. Measure the concentration of cycled tubulin using UV absorbance.

For normal rhodamine-labeled microtubules, the cycled tubulin and rhodamine-labeled tubulin are mixed to yield a dye:tubulin ratio of 1:5, and the solution is diluted using BRB80 buffer to a final concentration of 4 mg/ml. The solution is aliquotted into 10- $\mu$ l aliquots, flash-frozen in liquid nitrogen, and stored at -80°C for later use.

**Table 13.2** Microtubule Polymerization Recipe

Vol.	Reagent (Stock Conc.)	Final Conc.
10 $\mu$ l	Rho-tubulin (4 mg/ml, 1:5 rho:tubulin)	32 $\mu$ M
0.5 $\mu$ l	MgCl <sub>2</sub> (100 mM)	4 mM
0.5 $\mu$ l	MgGTP (25 mM)	1 mM
0.6 $\mu$ l	DMSO	5%
<u>0.9 <math>\mu</math>l</u>	BRB80	
Total	12.5 $\mu$ l	

#### 13.3.2.4 Polymerizing and stabilizing microtubules for microtubule gliding assays

To polymerize microtubules for kinesin motility experiments, thaw a 10- $\mu$ l aliquot of rhodamine tubulin and add the components listed in Table 13.2.

Mix by briefly flicking tube or pipetting, and incubate the solution at 37°C for 20 minutes to polymerize the microtubules. Meanwhile, make a 1-ml solution of BRB80 + 10- $\mu$ M Taxol (BRB80T) by adding 10  $\mu$ l of 1-mM Taxol in DMSO to 990- $\mu$ l BRB80 and rapidly vortexing to disperse the Taxol. Following polymerization, add the entire microtubule solution to the BRB80T solution and disperse by inverting the tube repeatedly. This results in the Taxol binding to the microtubules and stabilizing in their polymer form. These microtubules (called MT100 for 100-fold diluted microtubules) are stable for a week on the bench at room temperature. However, placing the tube on ice for a few minutes will depolymerize the microtubules. This microtubule solution can be flash-frozen on liquid nitrogen and stored at -80°C; thaw rapidly (warm hands work well) to prevent depolymerization.

### 13.3.3 Standard protocol for the microtubule gliding assay

The microtubule gliding assay involves immobilizing kinesin motors on a glass coverslip, introducing fluorescent microtubules in the presence of ATP, and observing their kinesin-driven transport. Versions of this assay are published in [14, 15] and online (<http://www.cellbio.duke.edu/kinesin/>), and there are many potential modifications that can be made. This assay is included here because it provides the foundational experiments for achieving kinesin-driven transport in microfluidic devices. It is also routinely used as a control experiment to assess the function of motors, microtubules, and other reagents. The protocol for the microtubule gliding assay are as follows:

1. Prepare a flow cell using a glass microscope slide and 1½ coverslip, using double-stick tape as a spacer. The depth of the cell will be ~100  $\mu$ m, and the width can be set by where the tape is placed.
2. Prepare 1 ml each of three solutions. BRB80CS is a casein-blocking solution consisting of 0.5-mg/ml filtered casein in BRB80. BRB80CA, made of 0.2-mg/ml casein and 1-mM ATP in BRB80 is used for motor dilutions. BRB80CT, made of 0.2-mg/ml casein and 10- $\mu$ M paclitaxel in BRB80 is used for microtubule solutions.
2. Block the surface by flowing 50  $\mu$ l of BRB80CS into the flow cell and incubating for 5 minutes. By carefully pipetting the solution into the edge of the experimental chamber, capillary action will draw the solution into the flow cell, filling it. The casein adsorbs to the glass surface and prevents denaturation of the motors on the surface.

3. Dilute the purified kinesin to 1  $\mu\text{g/ml}$  in BRB80CA, and introduce 50  $\mu\text{l}$  of this motor solution into the flow cell. Solution exchange is achieved by introducing the solution into one end of the flow cell using a pipette while simultaneously wicking the solution out of the other end of the flow cell using a Kim Wipe, filter paper, or other absorbent material. Incubate for 5 minutes to allow motor binding to the glass surface. (*Note:* Useful starting motor concentrations are from 0.05 to 5  $\mu\text{g/ml}$ , and can be optimized empirically.)
4. For making motility solution, use stock solutions described in the “Materials” section with the recipe listed in Table 13.3.  
This motility solution contains microtubules, MgATP, Taxol to stabilize the microtubules, casein to maintain an adsorbed layer on the surface, and an oxygen scavenging system consisting of glucose, glucose oxidase, catalase, and  $\beta$ -mercaptoethanol to prevent photobleaching. Flush the flow cell with 50  $\mu\text{l}$  of this motility solution.
5. Observe microtubule movement using epifluorescence microscopy. It is possible to observe microtubule movements by eye, but optimal performance is achieved by using a CCD camera attached to the microscope.

### 13.3.4 Design considerations for integrating motor proteins into microfluidic devices

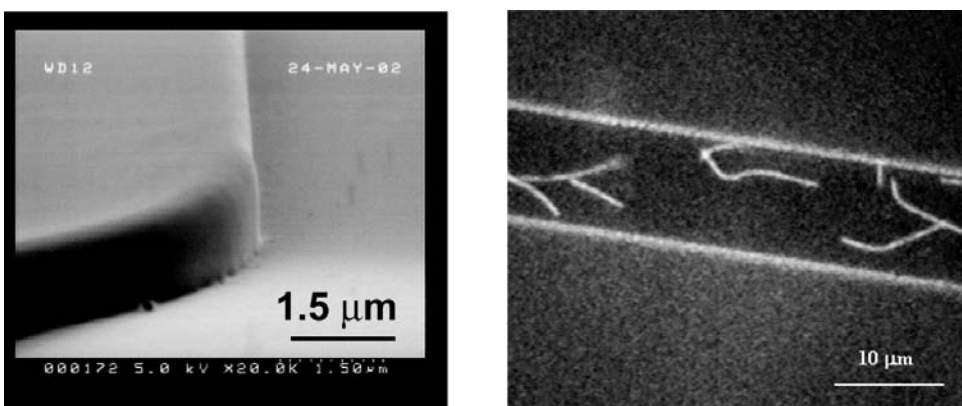
To successfully integrate motor proteins in microfluidic devices, the following issues need to be considered in the design of the device.

1. Compatibility of materials. The chosen materials should be transparent and support kinesin-driven motion. Some traditional photoresists such as 1811 are autofluorescent and as such they hinder the visualization of fluorescent microtubules. We have assessed the activity of kinesin motors on a range of surfaces and found that motors retain their function when adsorbed to a number of different hydrophilic surfaces including glass, gold and chrome electrodes, plasma-treated silicon, and plasma-treated SU-8. However, motors lose their function and/or denature on hydrophobic surfaces such as hydrophobic silanes and untreated SU-8 [16, 17]. In addition, some materials such PDMS cannot be used with fluorescent microtubules, as microtubules are photobleached and depolymerize under the microscope even in the presence of an antifade system. This behavior has been attributed to the high oxygen permeability of PDMS, which overwhelms the oxygen scavenging activity of the antifade system [18, 19].

**Table 13.3** Mobility Solution Recipe

Vol.	Reagent (Stock Conc.)	Final Conc.
85 $\mu\text{l}$	BRB80CT	
1 $\mu\text{l}$	MgATP (100 mM)	1 mM
1 $\mu\text{l}$	D-glucose (2 M)	20 mM
1 $\mu\text{l}$	glucose oxidase (2 mg/ml)	20 mg/ml
1 $\mu\text{l}$	catalase (0.8 mg/ml)	8 mg/ml
0.5 $\mu\text{l}$	$\beta$ -mercaptoethanol	0.5%
10 $\mu\text{l}$	MT100 (0.32 $\mu\text{M}$ )	~32 nM
100 $\mu\text{l}$		

2. Controlling microtubule movement. In the standard gliding assay, microtubules move in all directions across the surface, and the directionality is primarily determined by the orientation of the microtubules when they land on the surface. A number of groups have shown that microfabricated surface features such as walls and channels can be used to control the trajectory of kinesin-driven microtubules [17–22] (see Figure 13.2). One design feature that comes out of these studies is that controlling the direction of microtubule movement requires channels with widths less than  $10\ \mu\text{m}$  (the average length of microtubules polymerized in vitro). Due to the high flexural rigidity of microtubules, confinement in this size range forces the microtubules to follow the microchannel trajectory. Smaller channels provide better confinement, but control of fluid flow and solution exchange is increasingly challenging as the channel width is decreased.
3. Fluid flow and solution exchange. When enclosed microfluidic channels are used, fluidic connections from a syringe pump to the microchannels are required to enable the sample introduction into the device. The fluidic connections should be designed for fast solution exchange (the typical gliding assay requires two solution exchanges) and the fluidic connections should allow the sample to be mounted on a microscope stage.
4. Depletion of fuel and buffer. The surface of the typical gliding assay as described above is open to bulk solution so the  $\sim 100\text{-}\mu\text{m}$  layer of solution contains sufficient ATP to power the motors for many hours. However, in enclosed microchannels the high surface-to-volume ratio can lead to depletion of ATP fuel and loss in the buffering capacity of the solution over time [8]. Any design should therefore consider diffusion of these species into the channels, for instance by placing reservoirs along the length of the channel to replenish the fuel. Alternately, slow bulk flow in the channels can replenish the fuel without affecting microtubule movement.



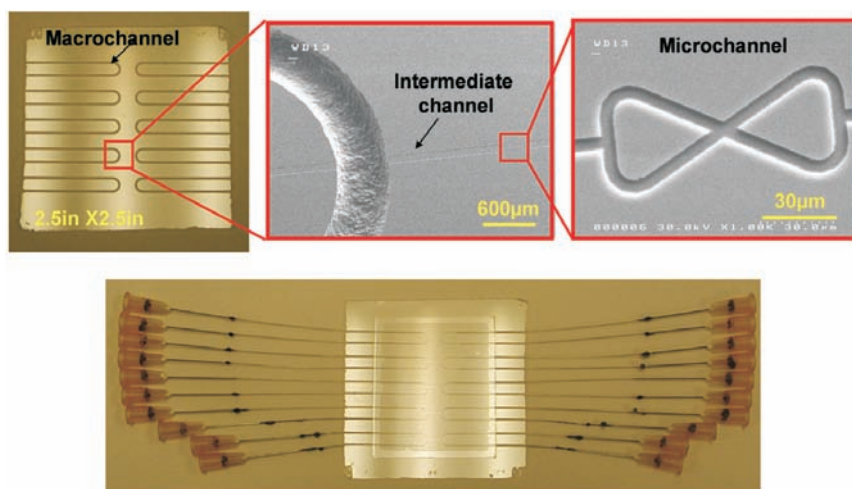
**Figure 13.2** Confinement of microtubule motion using microfabricated walls. SU-8 is deposited on a glass substrate to create micron-scale walls (left). Under the fluorescence microscope with simultaneous low-level bright field illumination, moving microtubules and the microchannel walls can be observed (right). Microtubules that collide with the walls buckle and are redirected, resulting in transport along the channel axis.

### 13.3.5 Fabricating enclosed glass channels for microtubule transport

We have developed an approach for fabricating microchannels that address all of the issues discussed in the previous section. The design consists of a three-tier hierarchical structure (Figure 13.3) that links functional microchannels to macroscopic fluid connections. Shallow microchannels ( $\sim 5\text{-}\mu\text{m}$  wide and  $\sim 1\text{-}\mu\text{m}$  deep) for microtubule confinement connect to intermediate channels ( $100\text{-}\mu\text{m}$  wide) that serve as reservoirs and also connect to  $250\text{-}\mu\text{m}$  deep macrochannels that hold fine gauge tubing for simple external fluid connections. The micro-, intermediate-, and macroscale channels are etched in a 1-mm thick borosilicate glass substrate and bonded to a cover glass using poly(methyl methacrylate) (PMMA) as an adhesive [8]. Figure 13.4 shows the processing steps involved in fabrication of the hierarchical channels.

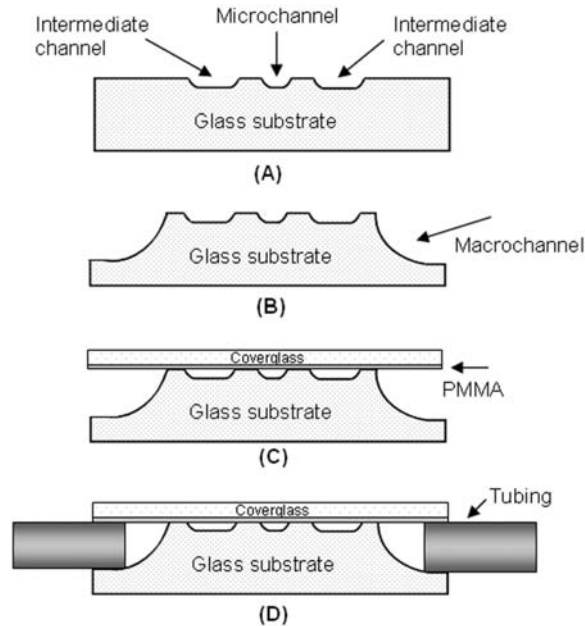
The materials used (glass and PMMA) support microtubule motility and do not interfere with visualization of fluorescent microtubules. PMMA is used as an adhesive to bond a glass coverslip to the glass substrate, a bonding approach that is more tolerant of particulates than anodic bonding or fusion bonding, and can be carried out at lower temperatures.

The macrochannel is designed in a U-shape with two tubing inserts connected to syringe needles to allow rapid solution exchange (See Figure 13.3). If a macrochannel with a single tubing is employed then each solution exchange requires a significant time to flush the solution present in the tubing and macrochannel through the narrow cross-section ( $1 \times 5\text{ }\mu\text{m}$ ) microchannel. Instead, in the U-shaped macrochannel one needle can be used to introduce solution and the other needle can be blocked to direct solution into the microchannel. For solution exchange, the second needle is unblocked to flush the U-shaped macrochannel with a new solution, and then is reblocked to direct the new solution into the microchannel.



**Figure 13.3** Hierarchical microchannel design. Macrochannels ( $250\text{-}\mu\text{m}$  deep) enable sample introduction through attached syringe needles. Intermediate channels ( $100\text{-}\mu\text{m}$  wide and  $1\text{-}\mu\text{m}$  deep) connect to microchannels ( $5\text{-}\mu\text{m}$  wide and  $1\text{-}\mu\text{m}$  deep) where microtubule motility is observed. The bottom panel shows a completed sample including the coverglass bonded using PMMA adhesive, and tubing for sample injection. (Image adapted, with kind permission from Springer Science+Business Media, from Huang et al. [8].)





**Figure 13.4** Schematic of fabrication process for enclosed glass microfluidic channels. Micro- and intermediate channels are first etched in the glass substrate (a), followed by etching deep macrochannels (b). A glass coverslip is then bonded using PMMA as an adhesive (c) and tubing for fluid connections are epoxied into the macrochannels (d). See text for fabrication details. (Image taken, with kind permission from Springer Science+Business Media, from Huang et al. [8].)

### 13.3.5.1 Microchannel fabrication protocol

The fabrication process is described below. For detailed protocols on making masks, photolithography steps, and photoresist development, consult the microfabrication protocols detailed in earlier chapters.

1. Clean glass substrates using acetone and isopropyl alcohol in an ultrasonic bath for 10 minutes each, followed by cleaning in Piranha solution ( $\text{H}_2\text{SO}_4:\text{H}_2\text{O}_2 = 4:1$ ) for 20 minutes. The Piranha treatment both cleans the glass and results in a high-surface energy OH-saturated surface that improves the adhesion of deposited layers.
2. Deposit a 10-nm Cr layer and a 100-nm Au layer on the cleaned glass substrate using a resistive filament thermal evaporator. Micro- and intermediate channel designs are then patterned on the metal layers by photolithography. First, spin on Shipley 1811 photoresist, then expose the sample through a mask (we use a Karl Suss MA-55 aligner), and finally develop the photoresist. (*Note:* The Cr/Au layer eliminates photoresist lifting that can be a problem for direct etching of glass with only a photoresist mask.)
3. Etch shallow micro- and intermediate channels using a buffered oxide etch solution ( $\text{NH}_4\text{F}/\text{HF}$  10:1) at room temperature with moderate agitation. A 20-minute etch results in 1- $\mu\text{m}$  deep channels, which work well for this application. After etching, strip the photoresist, Au, and Cr layers by immersing in acetone, Au etchant, and Cr etchant, respectively.
4. Etch U-shaped macroscale channels into the glass to connect the macroscopic fluid connections to the microchannels. The glass etching uses a robust Cr/Au/SU-8

protection layer and concentrated hydrofluoric acid (HF). First, deposit a thermally evaporated layer of Cr (10 nm) and Au (100 nm) to achieve good adhesion to the glass surface and provide a seed layer for gold electroplating. Second, deposit 1811 photoresist on this metal layer, pattern the U-shaped macrochannels in it by photolithography using a dark field mask, and then use Cr and Au etching to expose the glass in these regions. Third, electroplate a 1.5- $\mu\text{m}$  layer of gold (Techni gold 25ES, Technic Inc.) on the remaining Cr/Au. (*Note:* Active filtering of the electroplating solution during the electroplating process helps to remove particulates that can lead to pinhole defects in later steps.) Finally, spin on a 40- $\mu\text{m}$  layer of SU-8 photoresist, align to the U-shaped channels using a light field mask, and pattern the macrochannels in this SU-8 to expose the glass. The thick electroplated Au provides good protection against concentrated HF with reduced defect density and cost compared to evaporation or sputtering, and the SU-8 photoresist provides additional protection against pinholes.

5. Etch glass for 45 minutes in 49% HF at room temperature, which results in  $\sim 300\text{-}\mu\text{m}$  deep macrochannels. After etching, the SU-8 layer can be removed by piranha cleaning and the electroplated Au and evaporated Cr/Au layers removed by wet etching. Alternatively, the sample can be simply soaked in Cr etchant to lift off the whole Cr/Au/Au/SU-8 layer.
6. Spin-coat a 500-nm thick PMMA layer from chlorobenzene on  $48 \times 50\text{-mm}$  cover-glass (Gold seal #1, thickness: 0.13–0.17 mm) and bake on a  $180^\circ\text{C}$  hot plate for 20 minutes to remove the solvent and allow the PMMA layer to flow. Then, expose both the PMMA and glass microchannel surfaces to oxygen plasma for 1 minute to increase their adhesivity. Finally, laminate the PMMA-coated coverslip to the glass substrate using a Warner 100 hydraulic press laminator at 50 psi and  $120^\circ\text{C}$  for 10 minutes, and cool to room temperature while maintaining pressure. (*Note:* 996 kDa PMMA is chosen because it is sufficiently adhesive at convenient bonding temperatures to bond the two layers and is sufficiently viscous to prevent filling of the microchannels.)
7. Insert stainless-steel tubing into the macrochannels and bond using epoxy. Connect the other end of this tubing to syringe needles for sample introduction.

#### 13.3.5.2 Integrating motors and microtubules into microfluidic channels

The microtubule gliding assay protocol can be integrated into microfluidic channels by sequentially flowing casein, kinesin, and microtubule solutions into the channels. However, to account for the high surface-to-volume ratios in microchannels, the reagent concentrations should be increased. The high surface-to-volume ratio also leads to gradients in motor adsorption in the channels, with higher motor densities upstream and lower motor densities downstream due to depletion of motors. While this gradient can be eliminated by flowing in large amounts of kinesin solution to saturate the surface with motors, the resulting high motor densities in the intermediate channels lead to dense microtubule meshes forming at the macrochannel/intermediate channel junction that eventually clog the intermediate channels [8]. This problem can be solved using a headless kinesin construct [6] that competes with the full-length kinesin for surface adsorption to the channel walls but does not interact with microtubules. By varying the ratio of full-length kinesin to headless kinesin in the motor solution, an optimal surface

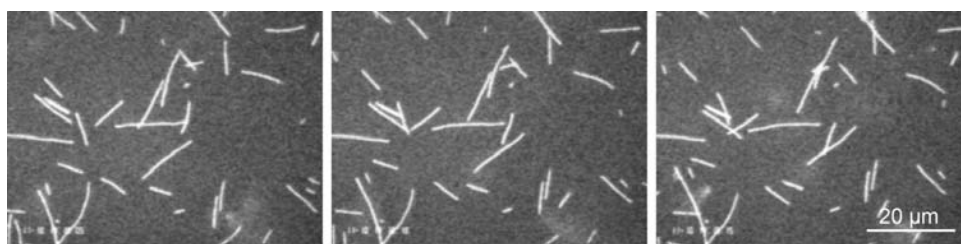
density of full-length kinesin can be obtained such that robust transport is achieved without dense networks of microtubules clogging the channels. The protocol for achieving functional microtubule transport in microfluidic channels is described below:

1. To immobilize motors inside the microchannels, introduce a solution containing 4-mg/ml casein, 7.5- $\mu$ g/ml conventional kinesin and 32- $\mu$ g/ml headless kinesin (KRT) in BRB80 buffer. (*Note:* Here we eliminated one solution exchange step by combining casein and kinesin solutions, using a large excess of casein such that casein adsorption will be much faster than kinesin adsorption. Alternately, casein solution can be introduced first, followed by the kinesin solution.)
2. Flush in a microtubule solution containing 0.64- $\mu$ M rhodamine-labeled microtubules, 10mM ATP, 50  $\mu$ M paclitaxel and antifade reagents (0.1 M D-glucose, 0.1 mg/ml glucose oxidase, 0.04mg/ml catalase, and 0.35M  $\beta$ -mercaptoethanol) in BRB80 buffer. (*Note:* The concentrations of ATP and antifade components are 10 to 20 times higher than standard assay to account for depletion in the microchannels.)
3. Visualize microtubule movements in the microchannels by epifluorescence microscopy. To simultaneously image the microchannels and the fluorescent microtubules, use simultaneous bright field microscopy with low illumination levels, which results in an overlaid image of the channels and the moving microtubules (as in Figure 13.2).

## 13.4 Results

Successful integration of microtubule-based motility into microfluidic channels requires both maintaining functional activity of the kinesin and microtubule proteins, as well as designing and fabricating biocompatible microchannels in geometries that generate useful transport. Here, we describe results from a standard microtubule gliding assay and then demonstrate two microchannel applications; one that generates unidirectional microtubule transport in enclosed channels and a second that accumulates dense bundles of uniformly aligned microtubules. These examples are from our published work [8], and for the interested reader there are a number of other published strategies from our group and others for controlling the kinesin-driven transport of microtubules in both open top and enclosed microchannels [1, 16, 17, 20–28].

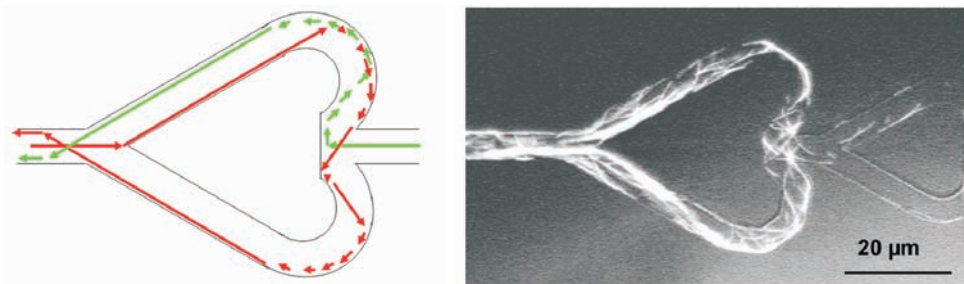
The microtubule gliding assay is both the experimental foundation for integrating microtubule transport into microfluidic channels, as well as a helpful research tool for assessing the activity of kinesin and microtubule proteins and for testing the



**Figure 13.5** Microtubules moving over a kinesin-coated surface in the microtubule gliding assay. Image acquired by fluorescence video microscopy with frames 5 seconds apart.

biocompatibility of different surface chemistries and materials. Figure 13.5 shows frames from a standard microtubule gliding assay, and a video is available on the Artech House Web site (<http://www.methodsinbioengineering.com>). Immediately following the introduction of microtubules, filaments land and move across the kinesin-covered surface in the range of 0.5 to 1  $\mu\text{m/s}$  at room temperature. Occasionally, microtubules will dissociate from the surface and diffuse away, but when high motor densities (1- $\mu\text{g/ml}$  kinesin and above) and long microtubules ( $> 5 \mu\text{m}$ ) are used this dissociation is rare. When very low kinesin concentrations are used in the assay, transient events can be observed in which microtubules land, are moved by individual kinesin motors, and then diffuse away when the motor reaches the minus-end of the microtubule [14]. Investigators who are exploring novel microchannel geometries, exploring new materials for such microchannels, or otherwise exploring this research area are advised to use the microtubule gliding assay to fully explore experimental variables before integrating the motility into channels.

When these same motors are integrated into enclosed microfluidic channels (fabricated according to the protocol in Section 3.5), the channel walls guide the microtubules and specific channel geometries can be used to achieve unidirectional microtubule transport. As a microtubule is being transported across a kinesin-functionalized surface, the leading end of the microtubule is continually encountering new motors. In the absence of any external forces the path the microtubule takes will tend to be fairly straight, since on these micron dimensions microtubules are quite stiff [29]. However, if the tip is biased to encounter motors in one specific direction, such as when a microtubule encounters a microfabricated barrier at an angle, the entire microtubule will tend to follow that path. One useful feature for microtubule-based microfluidic devices is a directional rectifier that causes microtubules to all move in the same direction. Figure 13.6 shows the design and performance of one such directional rectifier. Microtubules that enter from the left are guided either to the top or the bottom channel, and they travel around the structure and exit from the left. In contrast, microtubules that enter from the right collide with the channel wall, are guided either up or down, and then they continue through the channels and exit to the left. Thus, this structure generates uniform transport directions for a population of filaments. We found that



**Figure 13.6** A microtubule directional rectifier fabricated in enclosed glass microchannels. Schematic at left shows expected microtubule paths. Microtubules entering from the left (red trace) travel around the structure and exit to the left, while microtubules that enter from the right (green trace) pass through the structure. Image at right shows microtubules moving through the rectifier. Microtubules originate from a reservoir at left side of image; the small number of microtubules to the right of the structure demonstrate the successful redirection. (Image adapted, with kind permission from Springer Science+Business Media, from Huang et al. [8].)

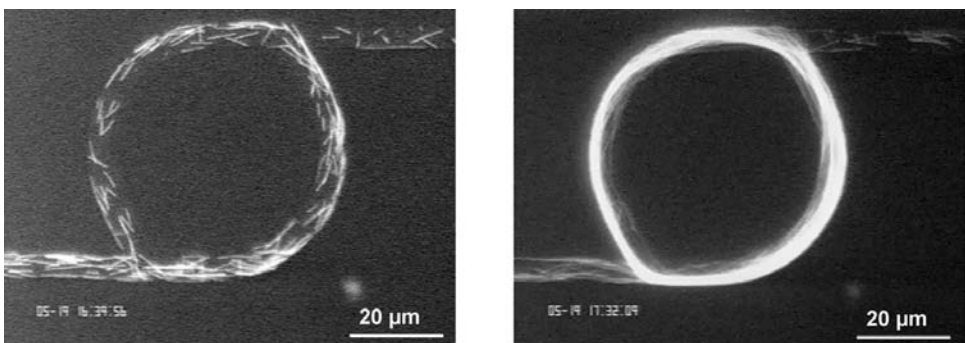
roughly 95% of the filaments are redirected in the correct orientation, and if higher tolerances are required, multiple rectifiers can be placed in series.

Another useful structure is one that both reorients microtubules to achieve uniform transport and acts to corral these microtubules to generate a collection of uniformly polarized filaments. We have accomplished this by fabricating enclosed glass microchannels to create a ring or “roundabout” structure. The fabrication approach detailed in Section 3.5 was used to create macrochannels for fluid introduction, intermediate channels that act as microtubule reservoirs (see Figure 13.3), and a circular microchannel was created that contains input and exit ports oriented tangentially (Figure 13.7). Microtubules that enter from the reservoir at the right travel around the ring, guided by the channel walls, while any filaments that change direction exit through one of the ports. Over time, thousands of uniformly oriented filaments can be collected that all move together through the channel. Among other uses, this system could serve as a starting point for cargo attachment, and if a gate were created, the filaments could be simultaneously directed at the chosen time into new channel.

The two microchannel structures described above are important building blocks towards realizing the device envisioned in Figure 13.1. There is clearly room for further developments in this area, and the successful strategies presented here can be considered a starting point for future studies in this area. In other work, we and others have developed approaches for attaching molecular beacons [4], magnetic nanoparticles [30, 31], quantum dots [32], and antibodies [33] to microtubules for carrying cargo. Most of these strategies rely on using biotinylated tubulin and using the biotin binding protein streptavidin as a bridge to attach the cargo. Tubulin can be biotinylated using the same protocol as described above for rhodamine-labeling.

## 13.5 Discussion of pitfalls

While there is considerable potential for incorporating microtubule motility into microfluidic channels, because this interdisciplinary area relies on both maintaining



**Figure 13.7** Microtubule concentration and unidirectional transport in an enclosed glass microchannel ring structure. Microtubules enter from reservoirs at both the left and right and either travel around the ring or are guided out of the ring. At left, microtubule movements shortly after introduction into the channel. Microtubules move in both directions at this point. At right, an image of concentration ring after 90 minutes of accumulation. Microtubules are moving counterclockwise around the ring; fluorescence intensity measurements estimate that thousands of microtubules are accumulated. (Image adapted, with kind permission from Springer Science+Business Media, from Huang et al. [8].)

functional proteins and fabricating useful microdevices, there are a number of points in the process that can fail. A number of potential pitfalls and alternate strategies are discussed here, following the same sequence used in the “Materials” and “Methods” sections.

### 13.5.1 Kinesin purification

The bacterial expression and column purification of kinesin has a number of potential pitfalls, and the protocol generally requires a few runs to achieve success. In the bacterial work, standard sterile technique is important to prevent bacterial contaminations. In assessing protein expression, running SDS-PAGE gels of bacteria before and after inducing with IPTG is very helpful. As the protein is hexaHis tagged, it is possible to use commercial kits (Novagen is one source) for detecting His-tagged proteins. If a centrifuge is not available, the bacterial lysate can be clarified by passing through a 0.45-micron syringe filter (though multiple filters are generally needed due to clogging). For the chromatography steps, other chromatography systems besides the FPLC will work fine and it is even possible to carry out batch (centrifuge-based) purifications using the Ni-NTA resin. As with expression, using SDS-PAGE gels to assay the motor concentrations in the clarified lysate, column flow through, washes, and elution is very helpful for troubleshooting. Gels of the elution fractions will also show the relative purity and the final concentration of the motor sample.

Following purification, it is generally a good idea to assess motor function using the microtubule gliding assay. Because this assay is relatively quick and is sensitive, it can be used for very low concentration samples to determine if any motor is present below the threshold of gel detection. One mode of failure is that the purified motors are nonfunctional. It is important to remember that retaining kinesin functionality requires keeping some nucleotide in the buffers (including all column buffers) at all time (10- $\mu$ M MgATP minimum is a good rule of thumb). Besides the microtubule gliding assay, another method for assessing motor function is to carry out a microtubule binding step in the presence of AMP-PNP, a nucleotide analog that locks the motors onto microtubules. Motor function can be assessed by incubating motors and microtubules in 1 mM AMP-PNP, pelleting the sample in the Airfuge, and analyzing the pellet and supernatant by SDS-PAGE. Nonfunctional (nonbinding) motors will remain in the supernatant, while functional motors will pellet with the microtubules. If column-purified motors are nonfunctional, it is advised to repeat expression and test the motility of the clarified lysate. Although there are a number of bacterial proteins, bacteria have no microtubule motors and if motors are functional in the bacteria, this lysate should work in gliding assays.

### 13.5.2 Tubulin

The tubulin purification protocol involves a number of steps that can go awry and success requires determination and persistence. On the plus side, it is reasonably easy to troubleshoot and determine which steps fail. As discussed, using fresh brains transported rapidly from the slaughterhouse is important for achieving high yields. As an aside, we have noticed that yields are reduced in the summer, and a colleague Richard Cyr has also reported this seasonal variation. As cows are homeotherms, the reason for this is not at all clear, but if preps can be carried out in other seasons, that is ideal. It

should be noted that there have been unpublished reports of tubulin preps starting with frozen brains, but we have no experience with this.

The first hurdle of the tubulin prep is obtaining the first microtubule pellet (following polymerization of the clarified brain homogenate). These pellets should be clearly observable by eye and have a somewhat opalescent quality to them. Very small pellets in this step almost always spell doom for the rest of the purification. A second indicator of success is an increase in viscosity as the tubulin solutions polymerize. While these indicators are diagnostics and not fixes, they are very helpful for assessing if the prep is working. One step toward avoiding failure in the tubulin prep is to ensure that the GTP is good. Purchase from a reliable supplier and store properly.

When labeling tubulin, losses are unavoidable—a 10% yield from the thawed tubulin sample is routine. One way to avoid further losses is to maintain the tubulin concentration above its critical concentration of ~1 mg/ml. In fact, maintaining the tubulin concentration above 5 mg/ml is recommended. Because the reactive dyes are fairly unstable, care should be taken to use dry (ideally newly purchased) DMSO for dissolving the reactive dyes. Also, excessive labeling can reduce yields of active tubulin. The degree of labeling can be optimized by changing either the dye concentration or the incubation time, and different fluorophores may have different optimal labeling times and concentrations.

For both the tubulin prep and tubulin labeling, three important points to keep in mind are (1) start with sufficient amounts of tubulin, (2) reduce volumes through the procedures to maintain sufficiently high tubulin concentrations, and (3) work to minimize the time that tubulin is sitting on the bench or in an ice bucket as the protein activity falls over time. While losses in tubulin activity are unavoidable, the good news is that active tubulin can easily be recovered by cycling such as in the tubulin labeling protocol (Section 3.2.3, steps 1–3). By polymerizing the active tubulin and pelleting it, any non-functional tubulin can be removed from the sample and, because the resuspension volume can be chosen, this is also a way to concentrate tubulin samples.

### 13.5.3 Motility assays

When working properly, the microtubule gliding assay serves as an important tool for testing and optimizing motor and microtubule function for microchannel experiments. However, the microtubule gliding assay itself can fail for a number of reasons. First, it is important to start with a sufficient concentration of stabilized microtubules. The presence of microtubules in the MT100 sample can be assessed by dropping a few microliters of the solution onto a microscope slide, covering with a glass coverslip, and visualizing the microtubules in this “squash” by epifluorescence microscopy. The next crucial reagent in the experiment is casein. Casein must be prepared as described in Materials and stored frozen. Beware that casein solutions left on the bench for days serve as excellent incubators for bacteria (think spoiled milk). If there are questions with the casein, it is best to make it up new from powder. The third important component of the gliding assay is the antifade system. The enzymes scavenge dissolved oxygen in the solution and the reducing agent further prevents free radical formation resulting from illumination of the fluorophores by the microscope illumination. Failure of the antifade system is fairly easy to assess because motility quickly ceases and the microtubules photobleach and then fragment due to free radical attack on the proteins. This can occur at the edges of

the flow cell or near any bubbles in the flow cell. If this is air contact and is ruled out, the motility solution should be made from scratch using newly thawed aliquots of all antifade components. Note also that  $\beta$ -mercaptoethanol can oxidize over time and should be replaced roughly every 6 months.

Motility assays can fail in two ways—either no microtubules land on the surface or microtubules land on the surface but they don't move. With proper casein treatment, microtubules do not bind to the glass coverslip surface in the absence of motors, and so any bound microtubules can be attributed to kinesin activity. If, after 5 minutes no microtubules are seen on the surface, this generally means either that the motor concentration is low or the motor activity is low or absent. The first option can be tested by repeating with a higher motor concentration. One way to enhance the signal from a small population of active motors is to repeat the motility experiment with the modification that the ATP in the motility solution is replaced with AMP-PNP. This nonhydrolyzable ATP analog causes motors to bind to microtubules irreversibly, so any filaments that do land are stuck and they accumulate over time. If no microtubule binding is seen after 5 to 10 minutes in AMP-PNP, this means there is no motor activity and suggests that the motors are completely inactive.

Another mode of failure for kinesins is irreversible binding to microtubules. If these so-called “deadheads” are present in a motor sample, the gliding speed will generally be reduced, spiraling microtubules will be observed at high motor concentrations (due to the front end getting stuck), and over time an increasing amount of bound but immobile microtubules will be observed. In very poor motor samples, it is possible to obtain only microtubule binding and no microtubule movement; there is generally no hope of rescuing these samples. If kinesin samples have a small number of deadheads, the activity can be improved by simply using lower motor concentrations on the surface. An alternative step is to “clean up” the motors by incubating motors with 1- $\mu$ M microtubules in 1-mM ATP for 10 minutes, pelleting the microtubules in the Airfuge to remove any motors that irreversibly bind, and collecting the supernatant that should contain active motors. In conclusion, because achieving kinesin-driven motility in channels rests upon reliable kinesin and microtubule samples, it is worth spending time working with gliding assays to fully characterize the functional activities of the motors and microtubules.

#### 13.5.4 Motility in microchannels

Achieving robust microtubule transport in microfluidic channels requires proper introduction of solutions into the channels, proper adsorption of motors to the channel walls, and good functional activity of the kinesin motors and microtubules. Before introducing motor or microtubule samples, it is often worthwhile to introduce a solution of fluorescent dye or labeled protein such as rhodamine-BSA into the channels to confirm that the channels are not occluded, that they don't leak, and that solution can be introduced into all regions of the device. Pumping a solution that is very easy to visualize also confirms that the bonding process can withstand the hydrostatic pressures created by the pump or syringe used to introduce the sample.

As discussed above, motility in microchannels differs from the standard gliding assay in the high surface to volume ratios involved. For this reason, it is often important to increase not only the motor concentrations introduced into the channels but also the



### Troubleshooting Table

Problem	Explanation	Potential Solutions
Kinesin purification failed.	No kinesin expressed.	Do miniprep and plasmid digest to confirm plasmid is correct. Make sure BL21(DE3) cells are used for expression. Try motility assay with clarified lysate to look for any motor activity.
	No kinesin purified.	Confirm that Ni column is properly charged. Perform motility assay with elution peak using AMP-PNP to look for any microtubule binding. Confirm kinesin expression (above).
	Kinesin is inactive.	Confirm that there is at least 1- $\mu$ M MgATP in all column buffers and that expression was carried out at 20°C.
Tubulin prep failed.	Low yield or activity.	Minimize time from cow to blender and maintain on ice. Check GTP by using it to polymerize some control tubulin. Run gels of all steps to determine where protein was lost.
Tubulin labeling failed.	Tubulin is present but not labeled.	Buy new rhodamine-NHS dye. Start with new bottle of dry DMSO.
	Low or zero tubulin yield.	Decrease labeling time and/or label concentration to control at ~1 label/tubulin. Spec solutions to determine where protein lost.
Microtubule gliding assay fails.	No microtubules on surface.	Confirm that microtubules are present by visualizing squash (see text) or focusing into solution above surface. Confirm kinesin concentration and use > 1 $\mu$ g/ml. Replace ATP with AMP-PNP to check microtubule binding.
	Microtubules bind to surface but don't move.	Dilute motors to minimize dead head influence. Confirm that ATP is present. Confirm that antifade is working and that $\beta$ ME is < 6 months old (will likely see fast bleaching). May need to do new kinesin prep to obtain functional motors.
No motility in microchannels.	Microtubules aren't seen.	Flow through dye to confirm channels are open. Visualize upstream to confirm microtubules aren't trapped. Use headless kinesin at entry to minimize microtubule sticking.
	Microtubules don't move.	Use higher kinesin concentrations to compensate for large surface area/volume. Increase concentration of antifade components. Use standard gliding assay to confirm that all reagents are working properly.

casein, ATP, and antifade concentrations (increases up to 10 $\times$  generally don't have any detrimental effects). As noted in Section 3.5.2, when high concentrations of kinesin adsorb to long channels, this can prevent microtubules from entering the channels because the microtubules bind and become tangled at the channel entry. Combining the active kinesin with headless kinesin reduces the maximal kinesin surface activity and helps to alleviate this problem. The optimum active:headless kinesin ratio is best determined empirically.

Generally, troubleshooting problems with kinesin-driven motility in channels is best achieved using the standard microtubule gliding assay. For instance, if novel substrates or photoresists are being used to create the microfluidic channels, flat surfaces can be created from these materials (such as by spinning the material onto coverslips), flow cells can be assembled on them, and the gliding assay can be carried out on the

modified surface. We have found that for a range of surfaces, plasma treatment renders them hydrophilic and compatible with kinesin motors.

### 13.5.5 Final comments

The integration of biologically-driven transport into microengineered devices is a relatively new and emerging area. To date, applications have focused on microscale transport in channels, but there are a number of potential directions for this research. Here, we have provided the foundation protocols for harvesting and modifying kinesin motors and microtubules as well as successful approaches for integrating these cellular proteins into engineered microdevices. These principles should provide a solid foundation for future investigations and will hopefully lead to functional devices based on these technologies.

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