Design of mechanical and electrical properties for multi-directional control of microtubules

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Running Head: Design of Microtubules for Multi-Directional Control

Abstract

Microtubule (MT)-motor systems show promise as nanoscale actuator platforms for performing molecular manipulations in nanobiotechnology and micro total analysis systems. These systems have been demonstrated to exert a variety of functions, including the concentration, transportation, and detection of molecular cargos. Although gliding direction control of MTs is necessary for these applications, most direction control methods are currently conducted using micro/nanofabricated guiding structures and/or flow, magnetic, and electric field forces. These control methods force all MTs to exhibit identical gliding behaviors and destinations. In this chapter, we describe an active multi-directional control method for MT without guiding tracks. The bottom-up molecular design allowed MTs to be guided in designated directions under an electric field in a microfluidic device. By designing the stiffness and surface charge density of MTs, three types of MT (*Stiff-MT*, *Soft-MT*, and *Charged soft-MT*) with different mechanical and electrical properties are prepared. The gliding directions within an electric field are predicted according to the measured stiffness and electrophoretic mobility. Finally, the *Stiff-MTs* are separated from *Soft-MTs* and *Charged soft-MTs* with a microfluidic sorter.

Keywords: microtubule, stiffness, electrical property, multi-directional control, trajectory prediction, sorting

1 Introduction

Microtubules (MTs) are one kind of polar, highly dynamic cytoskeleton filaments found in cells. They not only act as an intracellular transport system for motor proteins, but also play an important role in supporting cellular structures thanks to their mechanical properties [1, 2]. *In vitro*, MT-motor systems can be reconstituted as a molecular shuttle, or inversely MTs transported by the motor.

When combined with microfluidics technology, MT-motor systems can be used for molecular-scale delivery. Thus, these systems show promise as actuator platforms for use in nanobiotechnology research, including concentrators [3], transporters [4, 5], and detectors [5, 6]. However, the directional control of motor-driven MTs is a long-standing issue, due to the random gliding orientation of MT free leading tips as a result of the effects of Brownian motion. Despite the development of methods for the control of the gliding direction of these systems, including photoresist tracks [3, 7, 8], fluid shear force [9, 10], and magnetic and electric fields [11–14], driven MTs continue to be forced in a single direction with identical destinations, and the active control of the gliding direction of MTs remains difficult to achieve.

To address this challenge, this chapter reports on a multi-directional control method for MTs based on work conducted by Isozaki *et al*. [15, 16]. According to the cantilever beam model, the persistence of the MT gliding trajectory is proportional to its stiffness or persistence length (L_p). Additionally, MTs with different surface charge densities should have various electrophoretic mobilities, allowing them to glide in different directions in a given electric field [13, 14]. Thus, stiffer MTs with a lower surface charge density are more likely to glide with larger radii of curvature trajectories than softer MTs with a high surface charge density.

In this protocol, three types of MTs (*Stiff-MT*, *Soft-MT*, and *Charged soft-MT*) are designed and manipulated using their mechanical and electrical properties. After evaluating their stiffness and electrophoretic mobility, the gliding trajectories of the MTs are predicted (Fig. 1). Next, a microfluidic sorter is designed and fabricated. *Stiff-MTs* were separated from the *Soft-MTs* and *Charged soft-MTs* with an efficiency of 69.0% and 83.8%, respectively. The methods presented in this protocol, including the modification and evaluation of the properties (machinal and electrical) of MTs, multi-directional control, trajectory prediction, and sorting, can be generalized for other engineering applications.

2 Materials

Deionized water (DIW) with resistivity of ~18.2 MΩ·cm (at 25°C) is used for the solution preparation (unless stated otherwise). Dilute the stock solution to desired concentration shortly before assays. All the prepared proteins are stored in liquid nitrogen.

2.1 Reagents and solutions

1. 10 mM Guanosine 5′-[(α,β)-methylene] triphosphate (GMPCPP). Store at −20°C.
2. 0.1 M Guanosine 5′-triphosphate (GTP). Store at −20°C.
3. 1 M Dithiothreitol (DTT). Store at −20°C.
4. 4 mM Paclitaxel in dimethyl sulfoxide (DMSO). Store at −20°C.

5. 200 mM Succinimidyl ester-conjugated biotin in DMSO. Store at −20°C.
6. 6.0 mM Potassium Glutamate (K-glutamate). Store at 4°C.
7. 10 mM N-ethylmaleimide (NEM). Store at −20°C.
8. 14.3 M β-mercaptoethanol. Store at 4°C.
9. 2.0 mg/mL Streptavidin (SA). Store at −20°C.
10. 2.0 mg/mL Pluronic F108. Store at 4°C.
11. 10 mg/mL Casein. Store at 4°C.
12. 10 mg/mL Biotin-PEG-silane in 97% ethanol. Store at -20°C.
13. BRB80 buffer: 80 mM Piperazine-*N*, *N*′-bis(2-ethanesulfonic acid) (PIPES), 1 mM $MgCl_2$, and 1 mM Ethylene glycol-bis(2-aminoethylether)-*N*, *N*, *N*′, *N*′-tetraacetic acid (EGTA) (pH 6.8, adjusted with KOH) (*see* **Note 1**).
14. BRB80-O_2 buffer: 8.0 μg/mL Catalase, 20 μg/mL Glucose oxidase, 1 vol% β-mercaptoethanol, 20 mM DTT, 25 mM D-glucose, 10 vol% Glycerol, 15.8 μM Paclitaxel.
15. BRB80-O_2-ATP buffer: 0.50 mM ATP, 0.30 mg/mL Casein dissolved with BRB80-O_2 buffer.
16. 5′-biotinylated single-strand (ss-) DNA: 5′-Biotin- GAG GTC TTA ACG GTG GAG GAT GGG GGT TAG TCC GGG GCG CAG ATT CGA AT -3′.

17. Complementary 5′-AlexaFluor 488-tagged ssDNA: 5′- AlexaFluor 488-ATT CGA ATC TGC GCC CCG GAC TAA CCC CCA TCC TCC ACC GTT AAG ACC TC -3′.
18. The double-strand (ds-) DNAs: hybridized by incubating 10 μL of 5.0 μM 5′-biotinylated ssDNA and 10 μL of 5.0 μM complementary 5′-AlexaFluor 488-tagged ssDNA at a 1:1 molar ratio at 37°C for 20 min.
19. 6.1 mg/mL Non-labeled tubulin, 4.2 mg/mL Recycled tubulin, 6.0 mg/mL TAMRA-labeled tubulin, 5.8 mg/mL Alexa488-labelled tubulin (*see* Subheading **2.2** and **3.1**).
20. 0.3 mg/mL Kinesin (*see* Subheading **2.3** and **3.2**).
21. Hexamethyldisilazane. Store at 4°C.
22. SU-8 3010 photoresist. Store at 4°C.

2.2 Tubulin purification

1. Porcine brain: purchased from a local slaughterhouse just before use.
2. Washing solution: 0.24 M Sucrose, 1 mM $MgSO_4$, 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4.
3. PC buffer: 0.1 M PIPES−NaOH, 1 mM $MgSO_4$, 1 mM EGTA (pH 7.0, adjusted with NaOH).

4. 100 mM Phenylmethylsulfonyl fluoride (PMSF) in 10 mL ethanol. Store at −20°C.
5. 200 mM Tetramethylrhodamine (TAMRA) succinimidyl ester in DMSO. Store at −80°C.
6. 200 mM Alexa Fluor™ 488 succinimidyl ester in DMSO. Store at −80°C.

2.3 Kinesin purification

1. Host Bacteria: *Escherichia coli* (*E. coli*) Rosetta™ DE3 pLys-Novagene with plasmid of pET30K573. Store at −80°C.
2. 20 mg/mL Kanamycin. Store at −20°C.
3. 100 mM Isopropyl-thio-β-D-galactopyranoside (IPTG). Store at −20°C.
4. LB media: 0.5 g Trypton, 0.25 g yeast extract, and 0.5 g NaCl in 50 mL DIW.
5. TMP media: 20 g Trypton, 15 g yeast extract, 8 g NaCl, 1.8 g Glucose, and 2 g KH_2PO_4 in 1L DIW.
6. Washing buffer: 10 mM Tris acetate, 250 mM NaCl, 1 mM Magnesium acetate, 20 mM Imidazole acetate, 0.1 mM PMSF, 1 mM ATP, 0.05 vol% β-mercaptoethanol, 0.5 μg/mL Leupeptin, 1 μg/mL Pepstatin·Chymostatin, 70 μg/mL Tosylphenylalanyl chloromethyl ketone, and 75 μg/mL Tert-Amyl methyl ether.

7. Affinity resin: TALON Metal Affinity Resin.

2.4 Optical Equipment

1. Inverted epifluorescence microscope (IX73; Olympus, Tokyo, Japan) with 60× (NA 1.35) and 100× (NA 1.4) oil-immersion objectives.
2. Excitation filter (GFP/DsRed-A-OMF; Opto-Line International, Inc., USA), absorbance filter (A11400-04; Hamamatsu Photonics, Hamamatsu, Japan), and a ND6 filter with a shutter (VMM-D3; Uniblitz, Rochester).
3. Charge-coupled device camera (ORCA-D2; Hamamatsu Photonics) controlled by HCImage software (Hamamatsu Photonics).

3 Methods

3.1 Tubulin purification

Tubulin protein is obtained from porcine brains after two rounds of polymerization and depolymerization, and phosphocellulose chromatography [17].

1. Wash the porcine brain with Washing solution and weigh it.
2. Add PC buffer (with 1 mL/g of brain e.g., 1 mL of PC buffer for 1 g brain) with 1 mM ATP, 0.5 mM PMSF and 0.5 mM DTT to the brain and

homogenize brain with waring blender.

3. 1st round of polymerization: Add Glycerol (with half volume of homogenized brain) with 1.0 mM ATP, and 0.5 mM GTP to the homogenized brain.
4. Incubate at 37°C for 45 min.
5. Centrifuge the solution at 160,000 × g and 37°C for 30 min and collect precipitation.
6. 1st round of depolymerization: Resuspend collected precipitation with 60 mL PC buffer with 0.2 mM GTP and homogenize it in 4°C.
7. Centrifuge the solution at 124,000 × g and 2°C for 45 min after homogenization.
8. 2nd round of polymerization and depolymerization: repeat step 2–5.
9. Flow the obtained solution through an ion exchange column to get final non-labeled tubulin.

Recycled tubulin is purified from the non-labeled tubulin with one more round of step 2–5 [17, 18]. TAMRA-labeled tubulin and Alexa488-labelled tubulin are obtained by labelling tubulin with TAMRA or AlexaFluor™ 488 succinimidyl ester, respectively [19].

3.2 Kinesin purification

Human kinesin (amino acid residues 1–573) with an N-terminal histidine tag expressed by pETK573 vector in *E. coli* is purified as previously described [20].

1. Culture the *E. coli* in 50 mL LB media containing 20 μg/mL Kanamycin (e.g., add 50 μL of 20 mg/mL Kanamycin) at 37°C, ~14 hours.
2. Transfer the *E. coli* media solution into 1 L of TMP media containing 20 μg/mL Kanamycin (e.g., add 1 mL of 20 mg/mL Kanamycin).
3. Continue to incubate the *E. coli* media solution at 37°C until the OD600 value of solution reaches to 0.5.
4. Add 0.2 mM IPTG (e.g., add 2 mL of 100 mM IPTG) to the media solution and incubate at 20°C, ~10 hours.
5. Centrifuge the culture media at 4,000 × g and 4°C for ~7 min to collect cell body.
6. Resuspend the cell body pellets in the washing buffer and centrifuge at 6,000 × g and 4°C for ~10 min.
7. Collect the cell body and lyse it by sonication for ~5min.
8. Centrifuge the resuspended solution at 340,000 × g and 4°C for ~20 min and collect supernatant.

9. Purify the protein using affinity resin to obtain kinesin.

3.3 Design of MTs with different flexural rigidities

MTs are designed with different flexural rigidities by modifying their polymerizing conditions, including nucleotides and growth rate (by changing tubulin concentration) (Fig. 2A and 2B). Two types of MTs are designed and polymerized from a tubulin solution consisting of non-labeled, recycled, and TAMRA-labeled tubulins at a molar ratio of 1.3:0.4:1.

3.3.1 Slowly polymerized GMPCPP-MTs (*Stiff-MT*)

1. Polymerize MTs with 10 μM tubulin (e.g., add 1.2 μL of 40.9 μM non-labeled tubulin, 0.30 μL of 43.6 μM recycled tubulin and 0.70 μL of 54.5 μM TAMRA-labeled tubulin) in the presence of 1 mM GMPCPP (e.g., add 1.0 μL of 10 mM GMPCPP), 1 mM DTT (e.g., add 0.50 μL of 20 mM DTT) and 6.3 μL of BRB80 at 37°C for 30 min (Fig. 2A).
2. Add 10 μL of 40 μM Paclitaxel to stabilize the polymerized MTs.
3. Name the slowly GMPCPP-polymerized MTs as *Stiff-MT*.

3.3.2 Fast polymerized GTP-MTs (*Soft-MT*)

1. Polymerize MTs with 30 μM tubulin (e.g., add 3.5 μL of 40.9 μM non-labeled tubulin, 1.0 μL of 43.6 μM recycled tubulin and 2.0 μL of 54.5 μM TAMRA-

labeled tubulin) in the presence of 1 mM GTP (e.g., add 0.50 μL of 20 mM GTP), 1 mM $MgSO_4$ (e.g., add 0.50 μL of 20 mM $MgSO_4$), and 2.5 μL of BRB80 at 37°C for 30 min (Fig. 2B).

2. Add 10 μL of 40 μM Paclitaxel to stabilize the polymerized MTs.
3. Name the fast GTP-polymerized MTs as *Soft-MT*.

3.4 Design of MTs with different electrical properties

MTs with different electrical properties are designed to alter the surface charge density of the MT tips by labeling DNA molecules to MT tips (Fig. 2C).

3.4.1 Biotinylate *Soft-MT*

1. Shorten the lengths of the *Soft-MTs* (from Subheading **3.3.2**) by syringe actuation using a 30-G syringe with a needle diameter of 0.16 mm (Fig. 2B and 2C-1).
2. Biotinylate 8 μL shortened *Soft-MTs* by incubating with 40 μL of 600 μM Succinimidyl ester-conjugated biotin at 37°C for 30 min (Fig. 2C-1).
3. Quench the unreacted biotin by adding 160 μL of 6.0 mM K-glutamate at room temperature for 10 min.
4. Centrifuge at 163,000 × *g* and 27°C for 15 min.

5. Resuspend the precipitated biotinylated shortened *Soft-MTs* using 10 μL of 20 μM Paclitaxel.

3.4.2 Label Soft-MT with dsDNA (Charged soft-MT)

1. Prepare the NEM-treated tubulin solution by mixing 8 μL of 43.6 μM recycled tubulin, 1 μL of 10 mM NEM and 1 μL of 10 mM GTP at 4°C for 10 min.
2. Add 1 μL of 5.0 vol% β-mercaptoethanol to the solution and incubate at 4°C for 10 min.
3. Elongate 1.7 μL of biotinylated shortened *Soft-MTs* (from Subheading **3.4.1**) with the mixed tubulin solution (e.g., add 9.5 μL of 40.9 μM non-labeled tubulin, 1.6 μL of 32.9 μM NEM-tubulin and 1.1 μL of 54.5 μM TAMRA-labeled tubulin) in the presence of 1 mM $MgSO_4$ (e.g., add 0.80 μL of 20 mM $MgSO_4$), 1 mM GTP (e.g., add 0.80 μL of 20 mM GTP), and 1.4 μL of BRB80 at 37°C for 60 min (Fig. 2C-2).
4. Incubate 5.0 μL of biotinylated elongated *Soft-MTs* with 7.0 μL of 1.7 μM SA at room temperature for 15 min to obtain SA-labeled *Soft-MTs*.
5. Centrifuge the solution at 163,000 × g and 27°C for 15 min and resuspend the SA-labeled *Soft-MTs* with 12 μL of 20 μM Paclitaxel.

6. Incubate 8.0 μL of SA-labeled *Soft-MTs* with 16 μL of 5.0 μM dsDNA to obtain DNA-labeled *Soft-MTs* at 37°C for 20 min (Fig. 2C-3).
7. Name the DNA-labeled *Soft-MTs* as *Charged soft-MT*.

3.5 Evaluation of mechanical and electrical properties of MTs

The mechanical properties of MTs (*Stiff-MT* and *Soft-MT*) are evaluated by measuring their persistence length (L_p). Minus-end biotinylated (MB) MTs are used in the L_p measurement process (Fig. 3). The MB-MTs have two segments: one part is biotinylated and used to be immobilized onto a substrate via biotin-streptavidin bond, and the other part freely thermally fluctuates in an isolated flow chamber.

3.5.1 Preparation of biotinylated MTs

1. Polymerize short seed MTs by incubating 3.7 μL of 43.6 μM recycled tubulin and 1.5 μL of 54.5 μM Alexa488-labeld tubulin in the presence of 1 mM DTT (e.g., add 1.0 μL of 20 mM DTT), 1 mM GMPCPP (e.g., add 1.0 μL of 20 mM GMPCPP), and 3.3 μL of BRB80 at 37°C for 30 min (Fig. 3A).
2. Biotinylate, quench, centrifuge and stabilize 8 μL of seed MTs to obtain biotinylated (B-) seeds as Subheading **3.4.1 (2-4)** (Fig. 3A).

3. Shear the seed MTs solution by a 30-G syringe with a needle diameter of 0.16 mm.
4. Prepare the GMPCPP-polymerized MB-MTs (*B-Stiff-MT*) by incubating the B-seeds under the same conditions as *Stiff-MT* in Subheading **3.3.1** (Fig. 3B).
5. Prepare the GTP-polymerized MB-MTs (*B-Soft-MT*) by incubating the B-seeds under the same conditions as *Soft-MT* in Subheading **3.3.2** (Fig. 3B).

3.5.2 Coverslip cleaning

1. Clean the glass coverslips in acetone, isopropanol, and HNO_3, in this order.
2. Rinse the coverslips with deionized water (DIW) and dry with nitrogen gas.
3. Expose the coverslips to air plasma for 40 s at a flow rate of 50 sccm.
4. Immerse the coverslips in a solution of Biotin-PEG-silane (e.g., 1 mg/mL Biotin-PEG-silane and 30 mM HCl in 97% ethanol) in a nitrogen chamber overnight.
5. Rinse the coverslips with 97% ethanol and DIW and dry with nitrogen gas.
6. Store the coverslips at 4°C until use.

3.5.3 Flow chamber setup and L_p measurement

1. Construct a flow chamber by bonding biotin-coated coverslip and non-coated slides with 10-μm-thick double-sided tape.

2. Introduce 10 μL of 2 mg/mL SA and incubate for 3 min.
3. Wash the chambers with BRB80.
4. Introduce and incubate for 5 min to partially immobilize 10 μL of MB-MTs (*B-Stiff-MT* or *B-Soft-MT*) onto a glass substrate via biotin-streptavidin bindings (Fig. 3C).
5. Flush away non-immobilized MTs with 10 μL of BRB80-O_2 buffer.
6. Seal the chamber with nail polish.
7. Capture sequential fluorescent images of fluctuating MTs (Fig. 3D) (*see* **Note 2**).
8. Skeletonize and binarize the MT shapes using Gaussian fitting using the MT tracking software FIESTA (Fig. 3D) [21].
9. Derive the L_p of MT by equating thermal energy with the bending energy of the MTs using a customized MATLAB algorithm (Fig. 3E and 3F).

3.5.4 Evaluation of electrical properties of MTs

Electrophoretic mobility measurements are used to evaluate the electrical properties of *Charged soft-MT* (from Subheading **3.4.2**).

1. Dilute 1.0 μL of *Charged soft-MT* solution with 10.0 μL of 20 μM Paclitaxel.

2. Pressure-drive the MT dilution solution into a silica channel (26.0 × 1.4 × 5.0 mm, $l \times h \times w$).
3. Measure the electrophoretic mobilities of MTs via their velocities using the laser Doppler method [15].

3.6 MT sorting according to their electro/mechanical properties

The radii of the MT trajectory curvatures, R_{MT}, are measured according to the MT properties, L_p, and electrophoretic mobilities. Then, the position of the separation wall is determined according to the measured R_{MT} (Fig. 4A). The MT sorting device is designed with three separate parts: MT loading area, alignment area, and sorting area (Fig. 4B) (*see* **Note 3**). Polydimethylsiloxane (PDMS) microfluidic devices with 10 μm height are fabricated and used for R_{MT} measurement and MT sorting, respectively.

3.6.1 Device mold fabrication

1. Dehydrate a silicon wafer at 200°C for 5 min.
2. Spin-coat with hexamethyldisilazane at 500 rpm for 5 s and 3,000 rpm for 40 s.
3. Bake at 120°C for 5 min.
4. Spin-coat SU-8 3010 photoresist at 500 rpm for 10 s and 3,000 rpm for 30 s.

5. Bake the wafer at 65°C for 2 min, 95°C for 3 min, and 65°C for 1 min.
6. Transfer the channel pattern using a mask aligner with an exposure energy of 200 mJ/cm^2.
7. Bake the wafer at 65°C for 1 min, 95°C for 2 min, and 65°C for 3 min.
8. Cool the wafer at room temperature for 10 min.
9. Develop the pattern within a developer at 40°C for 3 min.
10. Rinse the mold with isopropyl alcohol at 40°C for 10 s.
11. Dry the mold with nitrogen gas.

3.6.2 PDMS device

1. Mix the PDMS prepolymer with a curing agent at a weight ratio of 10:1.
2. Cast the mixture onto a mold at a thickness of ~5 mm.
3. De-gas in a vacuum chamber for 30 min.
4. Cure at 80°C for 2 h.
5. Peel the cured PDMS from the mold.
6. Punch to make reservoirs.
7. Expose air plasma for 40 s at a flow rate of 50 sccm before bonding.

3.6.3 Coverslip cleaning

1. Immerse coverslips in 10 N KOH solution overnight.

2. Rinse twice by ultrasonication in DIW at room temperature for 20 min.
3. Immerse the coverslips in 20% ethanol solution at room temperature for 10 min.
4. Rinse twice by immersing in DIW.
5. Dry with nitrogen gas.
6. Expose to air plasma for 40 s at a flow rate of 50 sccm before bonding.

3.6.4 R_{MT} measurement and device design

1. Introduce 5 μL of BRB80 into the flow cell.
2. Introduce 5 μL of 2 mg/mL Pluronic F108, 0.08 mg/mL kinesin, and 1.0 mg/mL casein solution into the flow cell, successively.
3. Incubate for 5 min at room temperature, and then wash with BRB80.
4. Introduce 5 μL of BRB80-O_2 buffer to the flow cell.
5. Add 5 μL of one type of MT solution (*Stiff-MT*, *Soft-MT*, or *Charged soft-MT*) from reservoir c to d and incubate at room temperature for 5 min.
6. Remove the MT solution.
7. Wash and fill all the reservoirs and the flow cell with BRB80-O_2-ATP buffer.
8. Insert platinum electrodes with an electric field of 3 kV/m.

9. Set the MTs entering from the origin in the positive *y*-direction, and the electric field direction is in the negative *x*-direction. The MT trajectory is defined as (Fig. 4A) [16]:

$$y(x) = R_{MT} \cdot \arccos\left(e^{-x/R_{MT}}\right).$$

10. Track MTs at the leading tips using Mark2 software (NiCT, Kobe).
11. Measure the R_{MT} values of the three types of MTs (*see* **Note 4**).
12. Within the E of 3 kV/m, the calculated R_{MT} of *Stiff-MT*, *Soft-MT*, and *Charged soft-MT* are 84.6, 58.8, and 35.2 μm, respectively.
13. Set the separation wall at (x, y) = (≥70 μm, 70 μm) (Fig. 4A).

3.6.5 MT sorting within an electric field

1. Design the MT sorting device with a separation wall and three separated parts: MT loading area, alignment area, and sorting area (Fig. 4B) (*see* **Note 5**).
2. Fabricate and assemble the MT sorting device, as described in Subheading **3.6.1**~**3.6.3**.
3. Introduce BRB80 into a flow cell from the reservoir ***a***.
4. Introduce 5 μL of 2 mg/mL Pluronic F108, 0.08 mg/mL kinesin, and 1.0 mg/mL casein to the flow cell, sequentially.

5. Incubate for 5 min and wash using BRB80 buffer.
6. Introduce 5 μL of BRB80-O_2 buffer to reservoirs ***a*** and ***b***.
7. Introduce 5 μL of mixed MT solution (*Stiff-MT* and *Soft-MT* in Fig. 4C or *Stiff-MT* and *Charged soft-MT* in Fig. 4E) from reservoir ***c*** to ***d*** and incubate at room temperature for 5 min.
8. Remove the MT solution in reservoir ***c***.
9. Wash and fill all the reservoirs and the flow cell with BRB80-O_2-ATP buffer.
10. Immerse platinum electrodes in reservoirs ***a*** and ***b*** and an electric field of 3 kV/m.

3.6.6 Optical imaging and analysis

1. Observe MTs under an IX73 inverted epifluorescence microscope with an excitation filter, absorbance filter, charge-coupled device camera, and 60× (NA 1.35) and 100× (NA 1.4) oil-immersion objectives.
2. Set the exposure time to 100 ms (1 frame/s) using an ND6 filter with a shutter.
3. Store the optical images as sequential image files in TIFF format using HCImage software.
4. Define trajectories by tracking MT leading tips with ImageJ at 15-s intervals once they enter the sorting area.

4 Notes

1. BRB80 buffer is used for the dilution and suspension of proteins, unless otherwise stated.
2. Only the freely fluctuating segment of MTs is used to evaluate L_p.
3. The microfluidic channel used for R_{MT} measurement adopted a similar design as Fig. 4B except for the absence of the separation wall.
4. MTs gliding discontinuously or rotating on a pivot are excluded from R_{MT} measurement.
5. The width of the separation channel should be less than 1 mm to obtain a uniform electric field.

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Figures

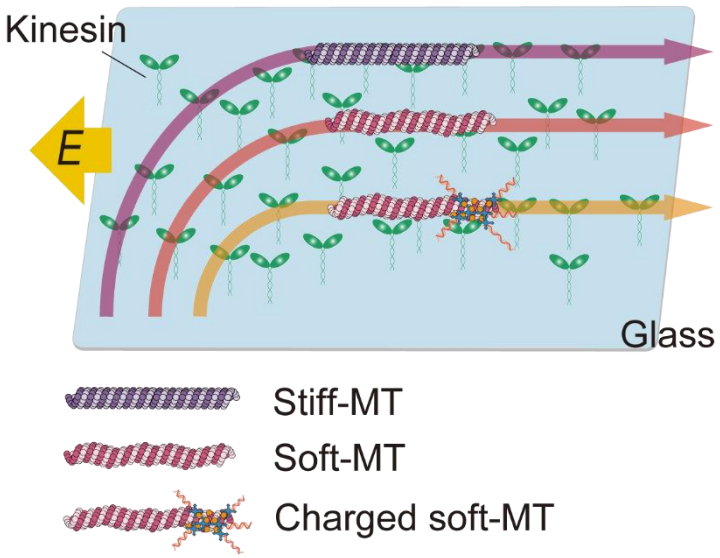

Fig. 1 Schematic representation of MT sorting under a given electric field. The gliding trajectories of kinesin-driven MTs are dependent on the mechanical and electrical properties of MTs. Three types of MTs are designed and polymerized under different conditions. Under a given electric field, *E*, MTs are guided to different destinations corresponding to their stiffness and surface charge density.

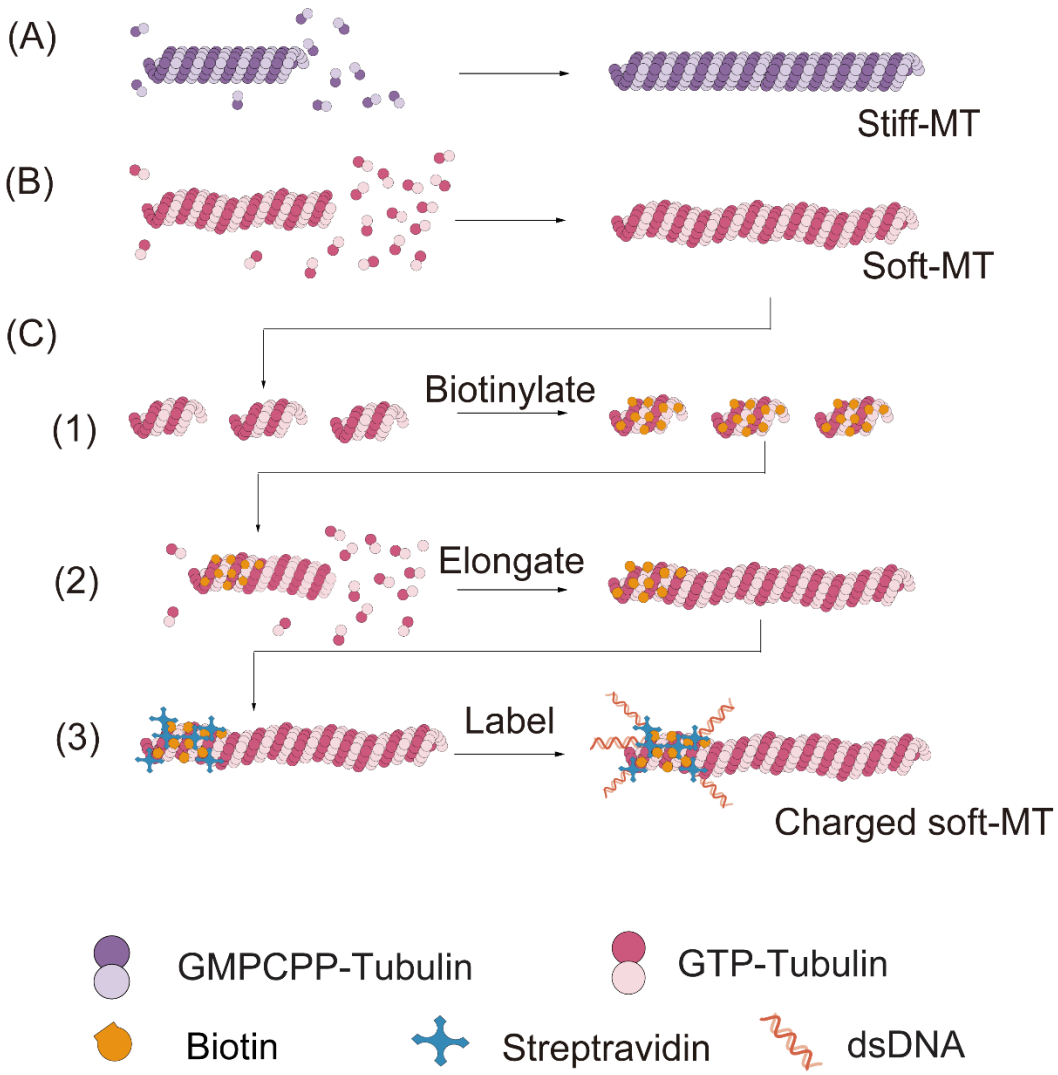

Fig. 2 Schematic illustration of the design of MTs. (A) *Stiff-MT* polymerized from 10 μM tubulin with GMPCPP. (B) *Soft-MT* polymerized from 30 μM tubulin with GTP. (C) *Soft-MT* is charged by labeling the minus end with dsDNA: (1) *Soft-MT* is shortened and biotinylated; (2) the biotinylated, shortened MTs are elongated within mixed tubulin solution; (3) the elongated MTs are labeled with SA and dsDNA in sequence to obtain *Charged soft-MT*.

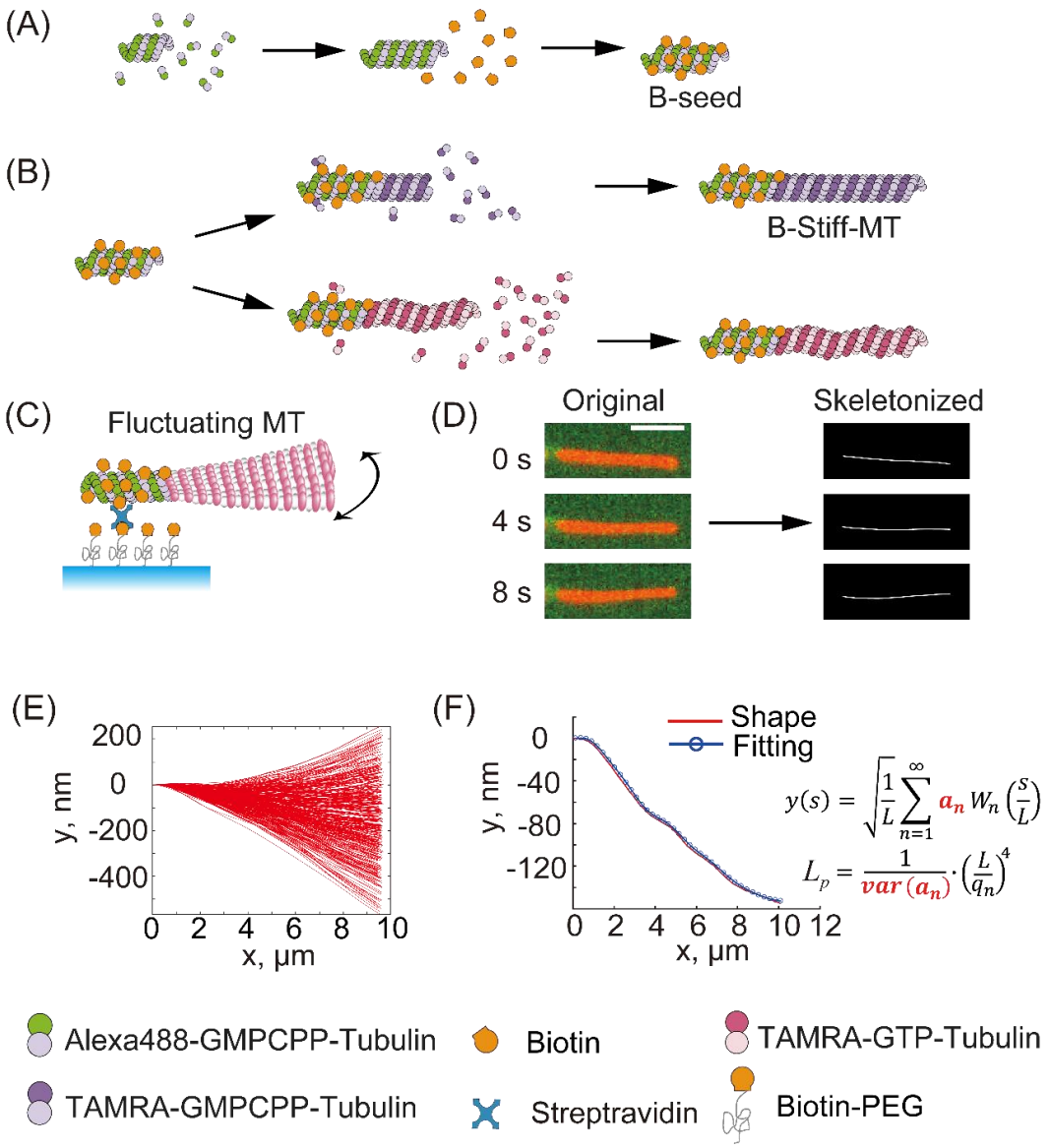

Fig. 3 Evaluation of the mechanical properties of MTs. (A) B-seed polymerized from Alexa488-labeled tubulin with GMPCPP and then biotinylated. (B) B-seeds are incubated in tubulin solution with GMPCPP or GTP to obtain MB-MTs (*B-Stiff-MT* or *B-Soft-MT*). (C) Biotinylated MTs immobilized via biotin-streptavidin bindings fluctuating under Brown motion. (D) the sequential fluctuating MT images are skeletonized and binarized using FIESTA. (E) Multiple sequential MT images are superposed. (F) The L_p of MT is derived using a custom MATLAB algorithm. Adapted from [16] with permission from Science Robotics.

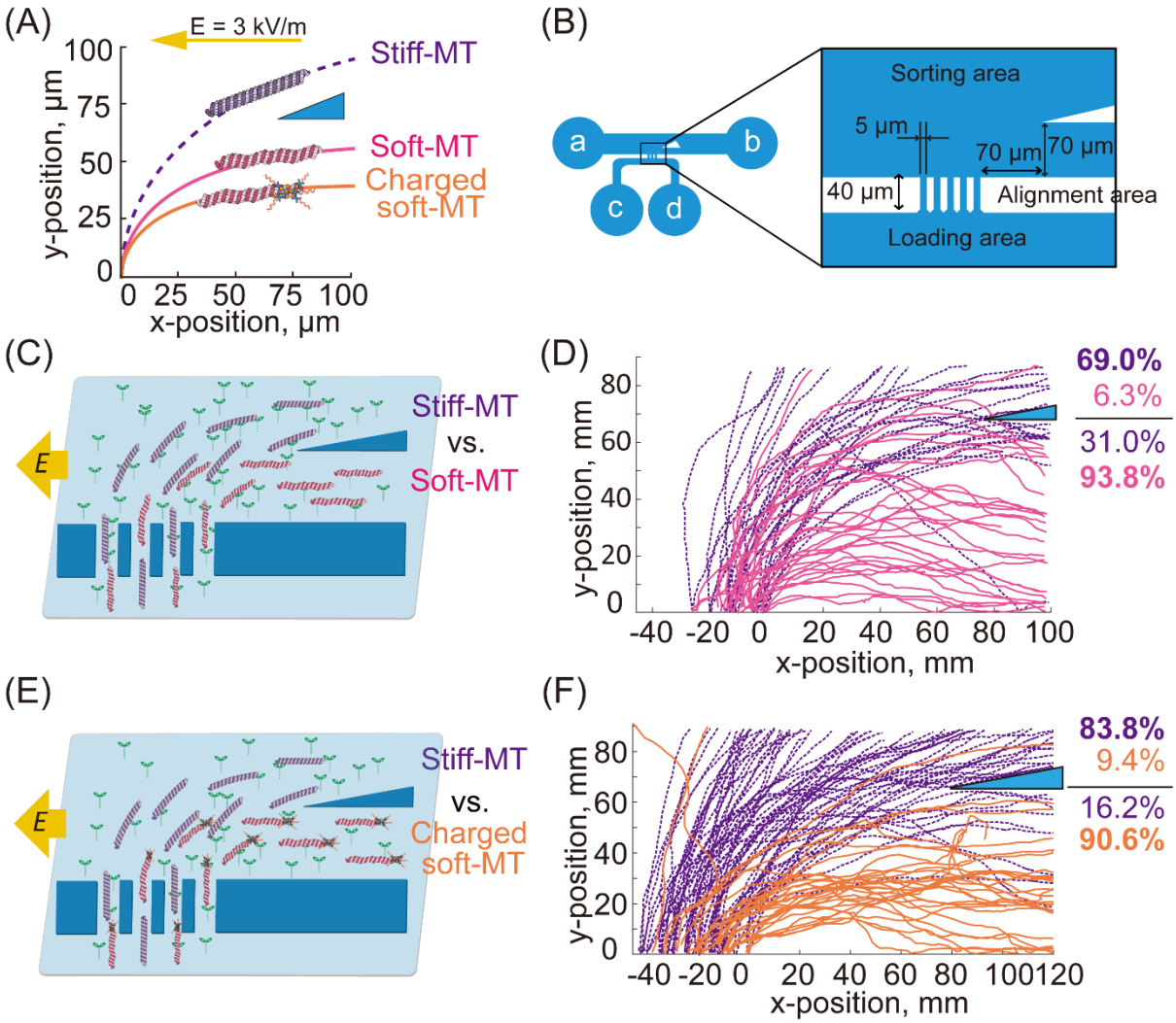

Fig. 4 Sorting of MTs using PDMS device within an electric field. (A) R_{MT} prediction and separation wall design. Predicted trajectories of *Stiff-MT* (purple, dashed line), *Soft-MT* (pink, solid line), and *Charged soft-MT* (orange, solid line) in the electric field of 3 kV/m. The separation wall is represented by a blue triangle. (B) Schematic representation of the PDMS device. The device consisted of three areas: MT sorting area (between reservoirs a and b), landing area (between reservoirs c and d), and alignment area (between MT landing and sorting areas). (C) *Stiff-MTs* are mixed and sorted from *Soft-MTs*. (D) *Stiff-MTs* (purple, dashed lines) and *Soft-MTs* (pink, solid lines) were sorted with an efficiency of 69.0% (n = 71). The blue triangle denotes the separation wall. (E) *Stiff-MTs* are mixed and sorted out from *Charged soft-MTs* and (F) *Stiff-MTs* (purple dashed lines) and *Charged soft-MTs* (orange solid lines) were sorted with an efficiency of 83.8% (n = 100). An electric field of 3 kV/m is applied in the negative *x*-direction. The upper

right corners of the MT alignment area are set to the origin. Adapted from [16] with permission from Science Robotics.