

Quantification of Spatial Patterns of Microtubule Transport by Kinesin-1 Head and Tail

Jashaswi Basu¹, Kajal Singh¹, Anita Jannasch² and Chaitanya A. Athale^{1, *}

¹Div. of Biology, IISER Pune, IISER Pune, Dr. Homi Bhabha Road, Pashan, Pune, India

²Center for Plant Molecular Biology (ZMBP), University of Tübingen, Tübingen, Germany

*For correspondence: cathale@iiserpune.ac.in

Abstract

The conventional kinesin-1 is a plus-end-directed microtubule-dependent motor protein with distinct motor head, stalk, and tail domains. Along with the motor head, which binds and walks along microtubules in an adenosine 5'-triphosphate (ATP) dependent manner, kinesin also contains a C-terminal microtubule binding tail. Motor-driven collective motility is well characterized using in vitro gliding assays, which show uninterrupted, smooth trajectories of transport. However, gliding assays driven by the full-length *Drosophila* kinesin-1 with both head and tail resulted in the emergence of spontaneous spatial microtubule patterns and stop-and-go motion. This was reproduced by an equimolar ratio of the active head and passive tail. Here, we describe the detailed protocol to reconstitute these microtubule gliding assays using multiple motor types: the full-length kinesin-1, the motor head or tail, mixtures of both head and tail, and a rigor mutant of the kinesin. We provide details of the approach taken to acquire the image time-series, to then quantify the spatial patterns that result from these motor combinations. Our approach provides a framework to systematically characterize the spatiotemporal effects of molecular motor-driven collective microtubule transport.

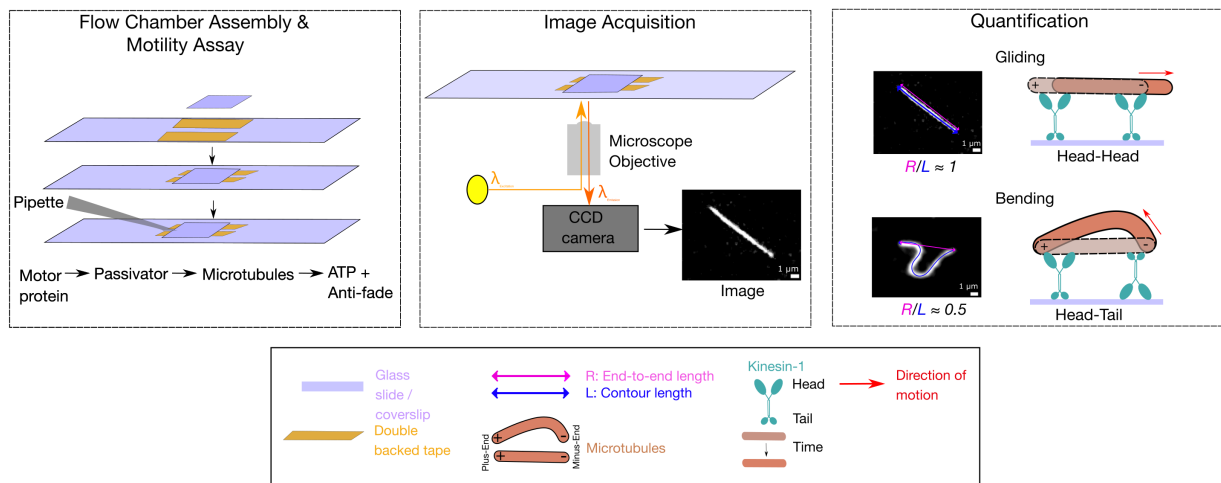
Key features

- This protocol highlights the cloning and expression of two major *Drosophila* kinesin-1 constructs: the microtubule binding tail and isoleucine-alanine-lysine (IAK)-deleted kinesin-1 full length.
- This protocol describes a typical gliding assay setup for the kinesin motor domain, alone as well as in combination with the kinesin tail.
- We present a systematic framework for typical gliding assays, including experimental acquisition as well as quantitative analysis of microtubule collective transport.
- This protocol gives a quantitative metric for microtubule spatial patterns, which enables systematic analysis of microtubule curvature, both in vitro and in vivo.

Keywords: Microtubule, Kinesin-1, Gliding assay, Microscopy, Image analysis, Gibson assembly

This protocol is used in: Molecular Biology of the Cell (2026), DOI: 10.1091/mbc.E25-09-0427

Graphical overview



Background

The plus-end-directed kinesin-1 is an essential microtubule-associated motor protein regulating intracellular transport. Kinesin-1 motor walks along microtubule filaments in an ATP-dependent manner, which is studied in vitro using gliding assays [1]. A classical gliding assay setup involves motor proteins immobilized on the surface, allowing microtubules to bind to motors. Force generated by the motor walk causes microtubules to glide in a direction opposite to the direction of motor walk. Microtubule motility driven by both kinesin [2] and dynein [3] can then be observed using fluorescence microscopy. Conventionally, motor-driven collective microtubule transport properties are identified using truncated motor domain constructs of kinesin-1 [4]. Interestingly, kinesin-1 full-length also consists of a C-terminal cargo binding tail [5], which can bind microtubules in an electrostatic manner [6] in an ATP-independent manner [7]. While motor-driven transport is well characterized, the role of the tail in gliding assays is less well known. Recently, we have shown that full-length *Drosophila* kinesin-1-driven gliding assays result in the emergence of spatiotemporal patterns such as bending, looping, and oscillations along with stop-and-go motion of microtubules [8]. These can be reproduced by an equimolar mixture of the active head and passive tail.

Similar spatial patterns have been reported both in cells [9] and modified gliding assays with artificial constraints for microtubules, resulting in patterns such as buckling [10], flagellar oscillation [11], and loops or spirals [12]. Thus, a systematic analysis of filament curvature, independent of the types of motion, helps us better understand microtubule mechanical properties, such as bending rigidity and persistence length. In previous work, the spontaneous bending patterns of microtubules in a thermal bath resulted in a measure of ~ 1 mm persistence length [13]. Motor forces exceeding 4–6 pN generated by kinesin in a gliding assay with one end of the filament clamped resulted in highly bent microtubules [10]. This has also been used to explain the highly curved microtubules inside cells as a result of the intracellular forces [14]. We have also observed such patterns in kinesin-1 gliding assays arising out of buckling instabilities due to the same filament being occasionally bound by an immobilizing tail and multiple active heads [8]. These patterns allow us to examine the effect of collective mechanics of both motors and microtubules in a simplified in vitro setup.

Here, we describe a gliding assay setup in order to explore spatial patterns in microtubules, driven by the activity of kinesin heads and tails. To this end, we have made a *Drosophila* kinesin-1 tail domain construct. In combination with the motor domain of kinesin-1, we have experimentally reconstituted gliding assays that result in bending patterns and quantified the spatial and temporal properties of microtubules. This approach of using gliding assays and quantitative analysis may be generally applicable to measure motor-driven cytoskeletal mechanics in vitro.

Materials and reagents

Biological materials

1. *Escherichia coli* DH5 α competent cells (The Coli Genetic Stock Center, Cheshire, CT, USA, Strain No: CGSC#: 14231)
2. *Escherichia coli* B121(DE3) competent cells (Thermo Fisher Scientific, USA, catalog number: EC00114)
3. K401, purified as previously described [15]
4. K980, purified as previously described [16]
5. Anti-GFP nanobody, purified as described [17]

Reagents

1. D(+)-Biotin (Sigma-Aldrich, catalog number: 8512090001)
2. 5(6)-Carboxytetramethylrhodamine N-succinimidyl ester (Sigma-Aldrich, catalog number: 21955)
3. Piperazine 1,4-bis (2-ethanesulphonic acid) (PIPES) (Sigma-Aldrich, catalog number: P6757-500G)
4. Magnesium chloride (Sigma-Aldrich, catalog number: 208337)
5. Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: E3889-100G)
6. GTP-disodium salt (Sigma-Aldrich, catalog number: 36051-31-7)
7. Adenosine 5'-triphosphate (ATP) disodium salt hydrate (Sigma-Aldrich, catalog number: A2383-10G)
8. Adenosine 5'-triphosphate (ATP) magnesium salt (Sigma-Aldrich, catalog number: A9187-1G)
9. 2-(N-Morpholino)ethanesulfonic acid (MES) hydrate (Sigma-Aldrich, catalog number: M8250- 250G)
10. Calcium chloride (Sigma-Aldrich, catalog number: C4901)
11. Paclitaxel (lyophilized powder) (Cytoskeleton Inc, catalog number: TXD01)
12. Casein sodium salt from bovine milk (Sigma-Aldrich, catalog number: C-8654)
13. Streptavidin, *Streptomyces avidinii* (Sigma-Aldrich, catalog number: 85878)
14. Sodium dihydrogen phosphate (Sigma-Aldrich, catalog number: 1.06370)
15. di-sodium hydrogen phosphate dihydrate (Sigma-Aldrich, catalog number: 1.06580)
16. β -Mercaptoethanol (Sigma-Aldrich, catalog number: 444203)
17. Phenyl methane sulfonyl fluoride (PMSF) (Sigma-Aldrich, catalog number: P7626)
18. Isopropyl- β -D-Thiogalactopyranoside (IPTG) [SRL Biochem, catalog number: 67208 (094866)]
19. Glucose oxidase (SRL Biochem, catalog number: 9001-37-0)
20. Catalase (SRL Biochem, catalog number: 9001-05-2)
21. D-(+)-Glucose (Sigma-Aldrich, catalog number: G5400-1KG)
22. Acetone (SRL Biochem, catalog number: 66951)
23. Potassium hydroxide (KOH) (Sigma-Aldrich, catalog number: 221473)
24. Bovine serum albumin (Sigma-Aldrich, catalog number: A7906-50G)
25. Glycerol (Sigma-Aldrich, catalog number: G901-2)
26. Luria Bertani (Lysogeny) broth, Miller (HiMedia, catalog number: G1245)
27. Tryptone (HiMedia Laboratories, catalog number: RM014-500G)
28. Yeast extract (HiMedia Laboratories, catalog number: RM027-500G)
29. Potassium phosphate dibasic (HiMedia Laboratories, catalog number: MB044-500G)
30. Potassium phosphate monobasic (HiMedia Laboratories, catalog number: MB050-500G)
31. NEBuilder[®] HiFi DNA Assembly master mix (New England Biology, catalog number: E2621S)
32. Q5[®] high-fidelity DNA polymerase (New England Biology, catalog number: M0491S)
33. Deoxynucleotide (dNTP) solution mix (New England Biology, catalog number: N0447L)
34. T4 polynucleotide kinase (New England Biology, catalog number: M0201S)
35. T4 DNA ligase (Promega, catalog number: M1801)
36. DpnI (New England Biology, catalog number: R0176S)
37. XbaI (New England Biolabs, catalog number: R0145)
38. Imidazole (Sigma-Aldrich, catalog number: 5710-OP)
39. HisPur[™] cobalt resin (Thermo Fisher Scientific, catalog number: 89965)
40. Ampicillin (Sigma-Aldrich, catalog number: A9518)
42. Rabbit anti-tetramethyl rhodamine antibody (Thermo Fisher Scientific, catalog number: A-6397)

43. QIAprep Spin Miniprep Kit (Qiagen, catalog number: 27104)
44. Pluronic F127 (Sigma-Aldrich, catalog number: P2443)
45. Primers
 - a. Forward primer for K910-980 (vector), ATGAACTATATAAAGGTACTAAACAGAAGATTTCTTC (Sigma-Aldrich)
 - b. Reverse primer for K910-980 (vector), AGTTCTTCTCCTTTGCTCATATGTATATCTCC (Sigma-Aldrich)
 - c. Forward primer for K910-980 (insert), TACATATGAGCAAAGGAGAAGAAGCTTTTCA (Sigma-Aldrich)
 - d. Reverse primer for K910-980 (insert), TGTTTAGTACCTTTATATAGTTTCATCCATGCCAT (Sigma-Aldrich)
 - e. Forward primer for K936, AACTCGCTTGTTCCGCGT (Sigma-Aldrich)
 - f. Reverse primer for K936, ACGTCTGCCCAGATGCTTC (Sigma-Aldrich)
46. Recombinant DNA plasmids
 - a. Drosophila Khc(1–980)-6xHis (K980), Addgene (unpublished), Addgene plasmid no. 129762, <https://www.addgene.org/129762/>
 - b. Drosophila Khc(1–401)-BCCP-6XHis (K401), Addgene [15], Addgene plasmid no. 15960, <https://www.addgene.org/15960/>
 - c. Human KIF5B(1–560)-GFP-6xHis (K560), Addgene [23], Addgene plasmid no. 15219, <https://www.addgene.org/15219/>
 - d. pGEX6P1-GFP-nanobody, Addgene [17], Addgene plasmid no. 61838, <https://www.addgene.org/61838/>

Solutions

1. 5× PIPES-EGTA-MgCl₂ (PEM) buffer (see Recipes)
2. K401 purification buffer (see Recipes)
3. K980/K560/KRigor purification buffer (see Recipes)
4. Taxol buffer (see Recipes)
5. Antifade mix (see Recipes)
6. Motility buffer (see Recipes)
7. PCR mix (see Recipes)
8. Gibson assembly mix (see Recipes)
9. Digestion–phosphorylation–ligation (DPL) buffer (see Recipes)
10. Digestion buffer (see Recipes)
11. High-molarity PIPES buffer (HMPB) (pH 6.8) (see Recipes)
12. Depolymerization buffer (pH 6.6) (see Recipes)
13. High pH cushion buffer (pH 8.6) (see Recipes)
14. Low pH cushion buffer (pH 8.6) (see Recipes)
15. Labeling buffer (pH 8.6) (see Recipes)
16. Quenching buffer (pH 7) (see Recipes)

Recipes

1. 5× PEM buffer (pH 6.8)

Reagent	Final concentration	Quantity or volume
PIPES	400 mM	6.048 g
EGTA	5 mM	0.951 g
MgCl ₂	25 mM	0.508 g
Double-distilled H ₂ O		Make up to 50 mL

In order to dissolve the components and achieve the desired pH, mix all components in a beaker, add a magnetic stirrer bead, and place the beaker on a rotational stirrer. Add a sufficient amount of KOH pellets, with constant stirring. Typically, we needed a 50 mL Falcon tube worth of KOH pellets. After the solution becomes clear, adjust the pH to 6.8 using more KOH. The solution can be stored at room temperature for at least 3 months.

2. K401 purification buffer

Reagent	Final concentration	Quantity or volume
200 mM Na-Phosphate (pH 7.2)	20 mM	10 mL
1 M β -mercaptoethanol	10 mM	1 mL
1 M $MgCl_2$	4 mM	0.4 mL
100 mM ATP	50 μ M	0.05 mL
50 mM PMSF	0.1 mM	0.2 mL
Double-distilled H_2O		Make up to 100 mL

Prepare the solution before use and keep at 0–4 °C for 30 min (freshly prepared).

3. K980/K560/KRigor purification buffer

Reagent	Final concentration	Quantity or volume
200 mM Na-Phosphate (pH 8)	50 mM	25 mL
5 M NaCl	300 mM	6 mL
100 mM β -mercaptoethanol	5 mM	0.5 mL
1 M $MgCl_2$	1 mM	0.1 mL
100 mM ATP	100 μ M	0.1 mL
50 mM PMSF	0.1 mM	0.2 mL
Double-distilled H_2O		Make the volume up to 100 mL

Prepare the solution at most 30 min before use and store at 0–4 °C until use.

4. Taxol buffer

Reagent	Final concentration	Quantity or volume
2 mM Paclitaxel	20 μ M	1 μ L
5 $\times$ PEM buffer	1 $\times$	20 μ L
Double-distilled H_2O		Make the volume up to 100 μ L

Prepare the solution and keep at 37 °C for 30 min before use.

5. 10 $\times$ antifade mix

Reagent	Final concentration	Quantity or volume
125 μ M glucose oxidase	10 μ M	8 μ L
40 μ M catalase	15 μ M	37.5 μ L
1 M glucose	500 mM	50 μ L
5 $\times$ PEM buffer	1 $\times$	20 μ L
Deionized H_2O		Make the volume up to 100 μ L

The solution can be prepared in advance and stored at -80 °C for ~1 month.

6. Motility buffer

Reagent	Final concentration	Quantity or volume
100 mM $MgCl_2$	5 mM	2 μ L
10 $\times$ antifade mix	1 $\times$	5 μ L
1,400 M β -mercaptoethanol	140 mM	25 μ L
2 mg/mL casein	0.5 mg/mL	12.5 μ L
100 mM MgATP	1 mM	2 μ L
5 $\times$ PEM buffer	1 $\times$	10 μ L
Double-distilled H_2O		Make up to 50 μ L

Prepare the solution and keep at 37 °C for 30 min before use (freshly prepared).

7. PCR mix

Reagent	Final concentration	Volume for 1 reaction
5 $\times$ Q5 reaction buffer	1 $\times$	10 μ L
10 mM DNTP mix	200 μ M	1 μ L
10 μ M forward primer	0.5 μ M	2.5 μ L

10 μ M reverse primer	0.5 μ M	2.5 μ L
Q5 high-fidelity DNA polymerase	0.02 U/ μ L	0.5 μ L
Template DNA	1 μ g	1 μ L
Autoclaved water		Make the volume up to 50 μ L

The PCR mix should be made for individual PCRs immediately before use. For each reaction, 50 μ L of total PCR mix was made.

8. Gibson assembly mix

Reagent	Final concentration	Volume for 1 reaction
0.05 pM vector	0.0125 pM	2.5 μ L
0.5 pM insert	0.1 pM	2 μ L
2 $\times$ NEBuilder HiFi DNA Assembly master mix	1 $\times$	5 μ L
Autoclaved double-distilled water		Make the volume up to 10 μ L

The mix should be made immediately before use. For each reaction, 10 μ L of assembly mix is needed.

9. DPL buffer

Reagent	Final concentration	Volume for 1 reaction
DNA	≤ 1 μ g	40 μ L
DpnI	0.02 U/ μ L	0.5 μ L
T4 kinase	0.2 U/ μ L	1 μ L
T4 ligase	0.01 U/ μ L	1 μ L
10 $\times$ T4 ligase buffer	1 $\times$	5 μ L
Autoclaved double-distilled water		Make up to 50 μ L

The mix should be made immediately before use. For each reaction, 50 μ L of the reaction mix is needed.

10. Digestion buffer

Reagent	Final concentration	Volume for 1 reaction
DNA	1 μ g	2 μ L
XbaI	0.4 U/ μ L	0.2 μ L
10 $\times$ CutSmart buffer	1 $\times$	1 μ L
Autoclaved double-distilled water		Make the volume up to 10 μ L

The mix should be made immediately before use. For each reaction, 10 μ L of digestion mix is needed.

11. HMPB (pH 6.9)

Reagent	Final concentration	Quantity or volume
PIPES	1 M	151.2 g
EGTA	10 mM	1.016 g
MgCl ₂	20 mM	3.804 g
Double-distilled H ₂ O		Make the volume up to 500 mL

In order to achieve the desired pH and dissolve the solutes, mix these components in a beaker, add a magnetic stirrer bead, and place on a rotational stirrer. Add a sufficient amount of KOH pellets, with constant stirring. Typically, we need a 50 mL Falcon tube worth of KOH pellets. After the solution becomes clear, adjust the pH to 6.9 using more KOH pellets. The solution should be made 16 h before tubulin purification and stored at 37 $^{\circ}$ C. This buffer should not be stored for more than a week.

12. Depolymerization buffer (pH 6.6)

Reagent	Final concentration	Quantity or volume
MES	50 mM	4.85 g
CaCl ₂	1 mM	0.073 g
Double-distilled H ₂ O		Make the volume up to 500 mL

In order to achieve the desired pH and dissolve the solutes, mix these components in a beaker, add a magnetic stirrer bead, and place on a rotational stirrer. Add sufficient amounts of HCl with constant stirring. The solution should be made 16 h before purification and stored overnight at 4 $^{\circ}$ C before use.

13. High pH cushion buffer (pH 8.6)

Reagent	Final concentration	Quantity or volume
HEPES	0.1 M	1.1915 g
EGTA	1 mM	19.02 g
MgCl ₂	1 mM	4.75 mg
Glycerol	60%	30 mL
Double-distilled H ₂ O		Make the volume up to 50 mL

In order to achieve the desired pH and dissolve the solutes, mix these components in a beaker, add a magnetic stirrer bead, and place on a rotational stirrer. Add a sufficient amount of NaOH pellets, with constant stirring. Typically, we need a 50 mL Falcon tube worth of NaOH pellets. After the solution becomes clear, adjust the pH to 8.6 using more NaOH pellets. The solution should be made 16 h before tubulin purification and stored at 37 °C. This buffer should not be stored for more than a week.

14. Low pH cushion buffer (pH 8.6)

Reagent	Final concentration	Quantity or volume
Glycerol	60%	6 mL
5× PEM	1×	2 mL
Double-distilled H ₂ O		Make the volume up to 10 mL

Store the buffer in 50 mL Falcon tubes at 37 °C overnight before use.

15. Labeling buffer (pH 8.6)

Reagent	Final concentration	Quantity or volume
HEPES	0.1 M	1.1915 g
EGTA	1 mM	19.02 g
MgCl ₂	1 mM	4.75 mg
Glycerol	40%	20 mL
Double-distilled H ₂ O		Make the volume up to 50 mL

In order to achieve the desired pH and dissolve the solutes, mix these components in a beaker, add a magnetic stirrer bead, and place on a rotational stirrer. Add a sufficient amount of NaOH pellets, with constant stirring. Typically, we need a 50 mL Falcon tube worth of NaOH pellets. After the solution becomes clear, adjust the pH to 8.6 using more NaOH pellets. The solution should be made 16 h before tubulin purification and stored at 37 °C. This buffer should not be stored for more than a week.

16. Quenching buffer (pH 7)

Reagent	Final concentration	Quantity or volume
K-glutamate	50 mM	0.0736 g
100 mM MgCl ₂	0.5 mM	1 mL
5× PEM buffer	1×	2 mL
Double-distilled H ₂ O		Make the volume up to 10 mL

The solution should be made 16 h before tubulin purification and stored at 4 °C. This buffer should not be stored for more than a week.

Laboratory supplies

1. Pipette tips: 2–20 µL (Tarsons, catalog number: 521000A), 10–100 µL (Tarsons, catalog number: 521010A), 100–1,000 µL (Tarsons, catalog number: 521020A)
2. Microtubes (microcentrifuge tubes): 500 µL (Tarsons, catalog number: 524060), 1,500 µL (Tarsons, catalog number: 524070A)
3. Ultracentrifuge polypropylene tube for TLA 100.3 ultracentrifuge rotor: 1.5 mL polypropylene tube (Beckmann Coulter, catalog number: 343169)
4. Ultracentrifuge polypropylene tube for TLA 120.2 ultracentrifuge rotor: 1 mL open-top thick-walled polypropylene tube (Beckmann Coulter, catalog number: 357656)
5. Ultracentrifuge tube for Ti 70 ultracentrifuge rotor: 26.3 mL polycarbonate bottle with cap assembly (dimensions: 25 ×

- 89 mm) (Beckmann Coulter, catalog number: 337922)
6. Micro cover glass 22 mm × 22 mm No. 1.5 (VWR, catalog number: 48366-227)
7. Glass slides 22 mm × 60 mm (HiMedia Laboratories, catalog number: BG003-5 × 50NO)
8. Double-sided Kapton[®] polyimide tape (Ted Pella Inc, catalog number: 16087-12)

Equipment

1. Pipettes: 1–10 µL, 2–20 µL, 20–200 µL, and 200–1,000 µL (PIPETMAN, Gilson)
2. Optima MAX-XP ultracentrifuge (Beckmann Coulter, model: 393315)
3. Optima XE-100 floor model ultracentrifuge (Beckmann Coulter, model: A94516)
4. Eppendorf high-speed centrifuge 5810R (Eppendorf, model: 5810R)
5. Benchtop ultracentrifuge rotor (Beckman Coulter, model: TLA 100.3)
6. Benchtop ultracentrifuge rotor (Beckman Coulter, model: TLA 120.2)
7. Floor model ultracentrifuge rotor (Beckman Coulter, model: Ti 70)
8. Eppendorf BioSpectrometerR basic (Eppendorf, catalog number: 6135HQ004308)
9. Thermomixer comfort dry heating block (Eppendorf)
10. Vibra Cell Ultrasonics (Sonics & Materials, Inc., model: VCX130)
11. Inverted epifluorescence microscope (Nikon Corp., model: Nikon TiE)
12. Lenses: CFI Plan Apochromat VC 60XA WI N.A. 1.20, W.D. 0.29 mm, CFI Plan Apochromat VC 100XH N.A.1.40, W.D. 0.13mm (Nikon Corp.)
13. Camera
14. Andor Clara2 CCD camera (Andor, Oxford Instruments)
15. Temperature-controlled chamber (Oko Labs)
16. Filters: TRITC filter: fC-FL Epi-Fl Filter Block TRITC, excitation: 385–425 nm; emission: 550–660 nm (Nikon Corp.); FITC filter: EGFP (FITC/Cy2) fluorescence filter, excitation: centered at 470 nm with a 40 nm bandwidth; emission: centered at 525 nm with a 45 nm bandwidth (Chroma Technology Corp)

Software and datasets

1. Fiji version 1.54p [18]
2. Python (version: v3.9.12) with packages SciPy (v1.7.3) [7] and NumPy (v1.21.5) [2]
3. MATLAB (version: R2022b)
4. FIESTA [1]
5. Inkscape 1.2 www.inkscape.org was used to generate vector graphics.

Procedure

A. Cloning, expression, and purification of recombinant kinesin tail

1. Prepare the PCR mix as described in the recipe with the forward and reverse primers (primer 1) specific to the K980 plasmid [Figure 1A (left)] and the K980 plasmid as template.
2. PCR-amplify the plasmid using the following conditions:
Initial denaturation: 2 min 98 °C
Cycle 1: ×10
Denaturation: 10 s 98 °C
Annealing: 20 s 60 °C
Extension: 120 s 72 °C
Cycle 2: ×25
Denaturation: 10 s 98 °C
Annealing: 20 s 64 °C

Extension: 120 s 72 °C

Final extension: 120 s 72 °C

3. Check the amplified DNA using agarose gel electrophoresis. The expected band should be between 4 and 5 kb as shown in Figure 1A (right).

4. Make the PCR mix as described with the forward and reverse primers (primer 2) specific to the K560 plasmid [Figure 1B (left)].

5. PCR-amplify the plasmid using the following conditions:

Initial denaturation: 2 min 98 °C

Cycle 1: ×10

Denaturation: 10 s 98 °C

Annealing: 20 s 58 °C

Extension: 21 s 72 °C

Cycle 2: ×20

Denaturation: 10 s 98 °C

Annealing: 20 s 63 °C

Extension: 21 s 72 °C

Final extension: 20 s 72 °C

6. Test the result of the PCR-amplified DNA using agarose gel electrophoresis. The expected band should be between 0.5 and 1 kb [Figure 1B (right)].

7. For both PCR-amplified products, proceed with DpnI digestion as follows: To 50 µL of PCR reaction, add 1 µL of the provided DpnI enzyme. Incubate the mix for 1 h at 37 °C. Follow this with the Gibson assembly after the digestion process.

Note: In order to minimize sample loss, proceed directly to Gibson assembly after DpnI digestion. Optionally, perform PCR cleanup using a commercial kit.

8. Gibson assembly

Note: Gibson assembly is an established cloning strategy to join two DNA fragments with overlapping ends [19]. In the following protocol, we describe the assembly strategy we followed to generate the plasmid encoding GFP-tagged kinesin tail domain. Here, we combine the nucleotide residues corresponding to the 910–980 amino acids of the kinesin-1 from the K980 plasmid (described in this study as vector) (Figure 1A) and the nucleotide residues corresponding to the GFP tag of the K560 plasmid (described in this study as insert) (Figure 1B).

a. Make a total volume of 10 µL.

b. Add the vector and insert to the NEB HiFi assembly buffer in a ratio of 1:3, as described in the Gibson assembly mix.

c. Incubate this Gibson assembly mix (G+) at 50 °C for 30 min.

d. After incubation, keep on ice for 5 min and proceed immediately for transformation.

Critical: Do not keep on ice for more than 10 min to obtain improved efficiency.

Note: As the Gibson negative (G-) control, prepare the Gibson reaction mix without the assembly buffer. Transform the negative control into E. coli DH5α cells to compare final colonies.

9. Transform both the assembly mix (G+) and negative control (G-) to competent E. coli DH5α cells by the heat shock method as follows:

a. Add 10 µL of the reaction mix to 50 µL of freshly thawed competent E. coli DH5α cells.

b. Incubate the mix at 4 °C for 30 min.

c. Heat-shock at 42 °C for 1 min followed by an immediate incubation at 4 °C for 5 min.

d. Add 1 mL of lysogeny broth and incubate at 37 °C and 180 rpm for 1 h.

e. After incubation, spread the mix on freshly made lysogeny agar plates supplemented with 100 µg/mL ampicillin.

f. Grow the cells overnight at 37 °C.

10. Check for colonies the next day. If you observe colonies in the agar plate with the G+ mix, proceed further for confirmation of assembly.

Critical: If assembly is successful, the G- plates should have no colonies. If there are, they should be significantly lower compared to G+. We suggest a fresh Gibson assembly for such cases. Assembly can be further verified by colony PCR of the transformant colonies in G+ plates, as described in step A9.

11. Pick individual colonies from the G+ plate and resuspend them in 50 μ L of sterile water. Boil the sample at 95 $^{\circ}$ C for 10 min. Spin the sample at 5,000 $\times$ g for 5 min and use the supernatant as the template for colony PCR as follows:

Initial denaturation: 2 min 98 $^{\circ}$ C

Cycle 1: $\times$ 30

Denaturation: 10 s 98 $^{\circ}$ C

Annealing: 25 s 67 $^{\circ}$ C

Extension: 200 s 72 $^{\circ}$ C

Final extension: 20 s 72 $^{\circ}$ C

12. Analyze the PCR-amplified products on a 1% agarose gel.

Note: We confirmed Gibson assembly of GFP-tagged tail domain construct, K910–980, using colony PCR with primers as described for the construct K936. For successful assembly, the amplified product should show a band at $\sim$ 4.6 kb, corresponding to the kinesin-1 tail construct. If colonies arise from residual template DNA either from the vector K980 or the insert K560, they may produce alternative band sizes either at $\sim$ 6.4 kb or no amplification at all, respectively. For additional verification, plasmid sequencing can be performed.

13. Isolate the plasmid using a column-based isolation kit (QIAprep Spin Miniprep kit).

14. To confirm the successful isolation of the plasmid of interest, add 2 μ L of the isolated DNA to the digestion reaction mix for a final volume of 10 μ L (see Recipes for digestion buffer) and incubate for 15 min at 37 $^{\circ}$ C.

15. Test the length of the digested plasmid by gel electrophoresis on a using 1% agarose gel. The expected size of the linearized fragment is 4.7 kb (Table 1), with the digested linearized plasmids typically migrating true to the length (comparable to the corresponding 5 kb molecular weight marker, MWM). The undigested circular and supercoiled plasmid migrates faster, due to differences in conformation, and appears closer to 5 kb (Figure 1C, right).

Note: If restriction digestion is not complete, resulting in a mixture of digested and undigested plasmids, one can try increasing the incubation time to 20 min.

16. Transform the assembled plasmid to *E. coli* BL21(DE3) cells for further expression and grow overnight at 37 $^{\circ}$ C in lysogeny broth (LB).

Note: Choose the LB media volume according to the required secondary culture volume for protein expression and purification. We chose 15 mL for 1,000 mL of secondary culture volume.

17. Inoculate 1,000 mL of LB with 10 mL of the primary culture (corresponding to 1% inoculum, v/v) supplemented with 100 μ g/mL ampicillin.

18. Incubate the culture at 37 $^{\circ}$ C with continuous shaking at 180 rpm.

19. Measure the optical density (OD) at 600 nm (OD_{600nm}) every hour using 1 mL samples of the growing culture in a plastic cuvette.

20. When OD_{600nm} = 0.4, add 0.4 mM IPTG.

21. Grow cells at 20 $^{\circ}$ C at 180 rpm for 5 h.

Caution: For optimal expression of soluble protein, do not grow the cells for more than 5 h.

22. Centrifuge the culture at 5,000 $\times$ g for 15 min to harvest the cells.

23. Resuspend the cells in 40 mL of lysis buffer and lyse by sonication (1 s on, 3 s off, with 60% amplitude) for 15–20 min.

Caution: Do not exceed 20 min and let the solution heat up. This might disrupt protein activity.

24. Centrifuge at 11,963 $\times$ g for 40 min to remove cellular debris.

25. Test protein expression by running both the supernatant and the pellet in 10% SDS-PAGE to check for a protein band of approximately 42.4 kDa.

26. Once confirmed, proceed with purification.

27. Incubate lysate supernatant with HisPur Co-NTA resin pre-equilibrated with K980 purification buffer at 4 $^{\circ}$ C for 1 h. For every 1 L of culture, use 3 mL of resin (RV, resin volume).

28. Remove flowthrough.

29. Wash the resin with 10 $\times$ RV K980 purification buffer (LyB).

30. Wash the column again with 5 $\times$ RV LyB buffer supplemented with 10 mM imidazole (pH 7) to elute out nonspecifically bound proteins.

31. Elute the tail domain protein using 2 mL of purification buffer supplemented with 200 mM imidazole (pH 7).

Note: For the first round of purification, try a range of imidazole concentrations. For Ni-NTA resins, the imidazole concentration might differ.

32. Check the eluted protein by running on a 10% SDS-PAGE. Expected molecular weight for the tail should be 42.4 kDa [Figure 1D (right)].

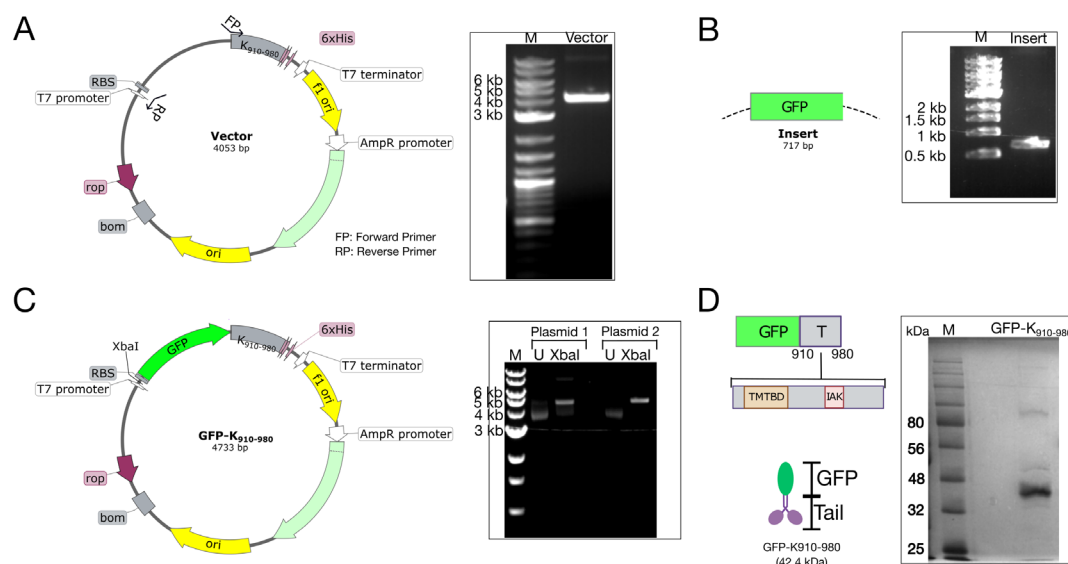


Figure 1. Cloning and expression of the GFP-tagged tail domain construct of *Drosophila* kinesin-1. (A) Representative gene map of the vector for Gibson assembly. The 4,053 bp long K980 plasmid (vector) was PCR amplified using primers that were complementary to the nucleotide residues coding for the Kinesin-1 tail domain, ranging between 910 and 980 amino acids (FP: forward primer; RP: reverse primer), and run on a 1% agarose gel (vector), along with the DNA ladder (M). (B) Representative gene map for the insert, PCR-amplified K560-GFP, with primers specific to the 710 bp long region encoding GFP (insert), run on a 1% agarose gel along with the DNA marker (M). (C) Representative gene map of the final assembled product, GFP-K910-980, with a size of 4.7 kb. Assembly was confirmed by restriction digestion using XbaI and run on a 1% agarose gel along with the undigested product (U), along with the DNA ladder (M). Plasmids 1 and 2 represent two independent isolates of the plasmid GFP-K910-980 construct from two separate DH5α transformants. (D) Schematic representation of domain structure of GFP-tagged tail, GFP-K910-980 (42.4 kDa), with 910-980 aa residues of full-length kinesin-1 consisting of both TMTBD (tail microtubule binding domain) and IAK (isoleucine-alanine-lysine) domains. The purified final product, GFP-K910-980, when run on a 10% SDS-PAGE, shows a distinct prominent band between 32 and 48 kDa. M: Molecular weight marker.

B. Cloning and purification of ΔIAK kinesin-1 full length construct (K936)

Note: The DNA constructs were designed as described in the Procedure and Figure 1. The protein constructs that we used in this study had domain structures related to K980 (Figure 2A), with K401 having only the essential head, neck, and stalk domain with a BCCP-tagged C-terminal (Figure 2B), the N-terminal GFP-tagged kinesin tail domain K910-980 (Figure 2C), and the ΔIAK construct K936 lacking the auto-inhibitory region (Figure 2D).

1. PCR-amplify the K980 plasmid using Q5 high-fidelity polymerase using forward and reverse primers as described for primer 3. The PCR conditions should be as follows:

Initial denaturation: 2 min 98 °C

Cycle 1: ×30

Denaturation: 10 s 98 °C

Annealing: 25 s 67 °C

Extension: 200 s 72 °C

Final extension: 20 s 72 °C

2. After PCR, run 5 μ L of the amplified DNA on a 1% agarose gel. The expected size should be $\sim$ 6.4 kb. If successful bands are observed, proceed with PCR clean-up.
3. Check the concentration of DNA. The ideal concentration for upstream processes should be $\leq$ 1 μ g.
4. To proceed with site-directed mutagenesis, add the amplified plasmid to the DPL buffer without T4 ligase, as described in the recipe, and incubate at 37 $^{\circ}$ C for 1 h. Add the T4 ligase enzyme and then incubate further for 1 h at 25 $^{\circ}$ C.
5. Transform the amplified product into *E. coli* Dh5 α cells, using the protocol described earlier for the tail construct (step A9).
6. For expression and purification, follow the protocol described for the tail domain (steps A16–32).

Table 1. Kinesin-1 construct details generated using the cloning strategy described in this study

Name	Full construct name	Plasmid size (kb)	Expressed protein molecular weight (kDa)	Source
K910-980	Drosophila GFP-Khc(910-980)-6xHis	4.733 kb	42.4	This study
K936	Drosophila Khc(1-936)-6xHis	6.449 kb	108.6	This study

C. Purification and labeling of tubulin

Note: Tubulin was isolated from goat brain using a temperature-dependent activity cycling-based protocol based on [20] with minor modifications following previously described methods [21,22].

C1. Activity-based purification of tubulin from goat brains

1. Obtain freshly slaughtered goat brain. Clean and weigh the brain tissue.
2. Check the mass of the brain. For every M g of brain mass, add M mL of de-polymerization buffer (DB).
3. Grind and homogenize the brain tissue until it turns into a bright red smoothie (tissue homogenate).
4. Centrifuge this homogenate at 29,000 $\times$ g for 1 h at 4 $^{\circ}$ C.

Note: We used the Ti 70 rotor in a tabletop ultracentrifuge for all the centrifugation steps during purification of brain tubulin.

5. Remove the supernatant and measure its volume. This is the supernatant volume (SV).

Note: Typically, for 80 g of tissue, we get 100–120 mL of supernatant.

6. Add 1 SV of warm high-molarity PIPES buffer, 1 SV of 100% glycerol, 1.5 mM ATP, and 0.5 mM GTP. Incubate the mixture at 37 $^{\circ}$ C for 1 h.
7. Spin the reaction by high-speed centrifugation at 150,000 $\times$ g for 30 min at 37 $^{\circ}$ C.
8. Remove the supernatant. Carefully mix 0.5 SV of cold DB (maintained at 4 $^{\circ}$ C) and incubate at 4 $^{\circ}$ C for 30 min.
9. Centrifuge the resuspended pellet at 70,000 $\times$ g for 30 min at 4 $^{\circ}$ C.
10. Measure the supernatant volume and repeat steps C1.6–7 (polymerization and centrifugation).

Note: The supernatant volume will decrease now. Adjust the volumes of HMPB and glycerol based on this modified SV.

11. After centrifugation, remove the supernatant carefully. Resuspend the pellet in 10 mL of chilled PEM buffer (stored at 4 $^{\circ}$ C) and incubate for 10 min to depolymerize.

Note: For 80 g of brain tissue, we normally add 5 mL of PEM buffer.

12. Centrifuge the resuspended pellet at 100,000 $\times$ g for 10 min at 4 $^{\circ}$ C. Store the supernatant at -80 $^{\circ}$ C after flash freezing it with liquid nitrogen.

C2. Labeling of brain tubulin

Note: Purified tubulin was labeled with carboxytetramethylrhodamine N-succinimidyl ester using a previously described protocol [18].

1. To the purified tubulin, add 3.5 mM MgCl₂, 1 mM GTP, and 30% glycerol and incubate for 1 h at 37 $^{\circ}$ C.

Note: We used 1 mL of 10 mg/mL of purified tubulin.

2. Add the polymerized tubulin to the high pH cushion buffer and centrifuge the sample at $278,700\times g$ for 45 min at 30 °C.
Note: The volumetric ratio of polymerized tubulin and cushion buffer should be maintained at 3:2. We used TLA 120.2 tubes with a capacity of 1 mL. For those tubes, 375 μ L of tubulin was added to 225 μ L of cushion buffer.

3. Aspirate the supernatant and cushion buffer. Wash the pellet with PEM buffer (maintained at 37 °C).

4. Resuspend the pellet with 800 μ L of warm labeling buffer (maintained at 37 °C).

5. Add 2.5 mM working concentration of the NHS ester (dissolved in DMSO) to the resuspended pellet. Keep the mixture at 37 °C with occasional stirring.

6. Repeat step C2.5.

7. Centrifuge the mixture by adding it on low pH cushion buffer at $278,700\times g$ for 20 min at 30 °C.

Note: A similar ratio of reaction mix and cushion buffer should be maintained.

8. Aspirate the supernatant and cushion carefully.

9. Resuspend the pellet with 600 μ L of chilled quenching buffer (maintained at 4 °C) to depolymerize and quench the labeling reaction by incubating for 30 min at 4 °C.

10. Centrifuge the depolymerized tubulin at $150,000\times g$ for 10 min at 4 °C. Collect the supernatant.

11. To the supernatant, add $MgCl_2$, GTP, and glycerol and incubate as in step C2.1.

12. Centrifuge the reaction and collect the pellet as in step C2.2.

13. Add 200 μ L of cold PEM buffer (maintained at 4 °C) to the pellet. Incubate the resuspended pellet for 30 min at 4 °C.

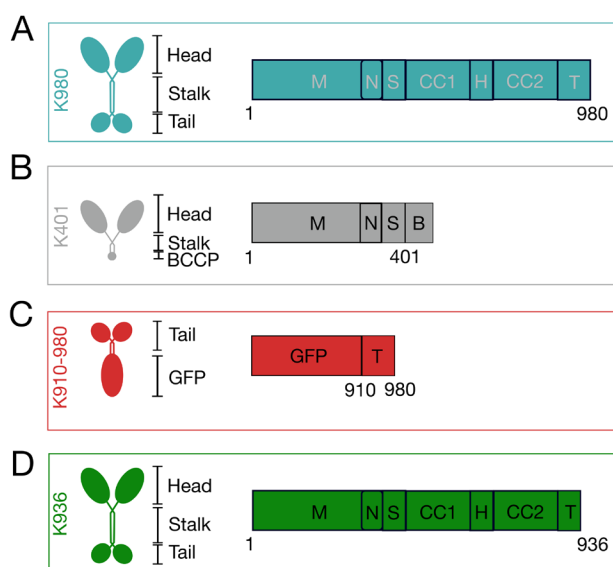


Figure 2. Schematic representing kinesin constructs used in this study. (A–D) Representative schematic to represent protein structure and domain organization of (A) full-length *Drosophila* kinesin-1, K980, (B) truncated minimal motor domain, K401, (C) truncated minimal tail domain, K910-980 (Table 1), and (D) full-length IAK deleted kinesin-1 mutant, K936 (Table 1) constructs. M: motor domain with ATP-binding site; N: neck domain; S: swivel; CC1/CC2: coiled coils 1 and 2; H: hinge; T: tail; B: C-terminal BCC19P tag present only in K401; GFP: GFP tag present only in K910-980.

D. In vitro microtubule gliding assays

D1. Microtubule filament assembly

1. Incubate a 20 μ L reaction mixture consisting of 30 μ M unlabeled tubulin, 5 μ M rhodamine-labeled tubulin, and 10% glycerol in PEM buffer at 37 °C for 30 min.

Note: The concentrations of labeled and unlabeled tubulin were chosen to achieve a labeled to unlabeled tubulin ratio of

1:6 based on previously described conditions [3]. This labeling ratio can be adjusted if required to achieve optimal fluorescence signal intensity.

2. Stabilize microtubule filaments with 100 μ L of taxol buffer. Mix the sample with a cutoff tip to avoid shearing of microtubules.

Critical: Use a cutoff pipette tip for each step that involves polymerized tubulin to avoid shearing of microtubules.

3. Remove free dimers by spinning them at 150,000 $\times$ g in a TLA 100.3 rotor for 15 min at 30 $^{\circ}$ C.

Note: If microtubules show a large number of aggregates, try centrifuging on a 60% glycerol solution in taxol buffer (cushion buffer). This acts as a cushion and reduces aggregates.

4. Aspirate the supernatant and the cushion carefully.

5. Resuspend the pellets in 100 μ L of taxol buffer. The pellets should look glassy and red due to the presence of rhodamine-labeled tubulin.

Note: Washing the pellets with 100 μ L of taxol buffer further removes any nonspecific aggregates.

6. Store the resuspended pellets in a 500 μ L tube covered with aluminum foil at 37 $^{\circ}$ C for further use.

Notes:

1. *Washing the pellets with 100 μ L of taxol buffer further removes any nonspecific aggregates.*

2. *This assembly protocol typically yields successful rhodamine-labeled microtubule filaments of lengths $\sim$ 2–15 μ m. This length range is not a strict requirement. The microtubule lengths are inspected in fluorescence microscopy (TRITC filter) by mounting a 2 μ L sample of the microtubule-containing suspension on a coverslip and inverting it on a slide for imaging.*

Critical: For optimal results in these gliding assays, maintain the average length of microtubules $\sim$ 2–15 μ m. Longer microtubules are more prone to bending and buckling [10], further contributing to variability in motility patterns. If longer filaments are observed, they can be shortened by gentle pipetting using 10 μ L pipette tips.

D2. Coverslip washing

Note: Coverslip washing with acetone and KOH is required for optimal and uniform protein binding on the coverslip glass surface.

1. Place coverslips of dimensions 22 mm $\times$ 22 mm in a 100 mL beaker.

2. Clean the coverslips with double-distilled water.

3. Add 50 mL of 100% acetone to the coverslips and keep them in a continuous shaker for 1 h.

4. Discard acetone and wash the coverslips thoroughly with double-distilled water to remove any traces of acetone.

5. Add 50 mL of 100% ethanol and keep them in a continuous shaker for 15 min.

6. Discard ethanol and wash the coverslips with double-distilled water to remove traces of ethanol.

7. Add 50 mL of 0.1 M KOH solution and keep them in a continuous shaker for 15 min.

8. Discard KOH and wash the coverslips with double-distilled water to remove traces of KOH.

Note: After washing, add fresh double-distilled water and check the pH of the solution. Proceed only if the pH is neutral.

9. Keep the coverslips in 100% isopropanol solution until use.

D3. Gliding assay

1. K980

a. Prepare flow chambers of 15 μ L (chamber volume) by sticking two parallel strips of double-backed Kapton polyimide tape to a glass slide (Figure 3).

b. Adhere acetone–KOH-washed 22 $\times$ 22 mm coverslips to the double-backed tape.

c. Flow 1 $\times$ chamber volume of 1 μ M of K980 to the glass chamber and incubate for 20 min at 25 $^{\circ}$ C.

d. Wash the flow chamber using a warm PEM buffer (maintained at 37 $^{\circ}$ C).

e. Repeat steps D3.1c–d three times to ensure maximum protein binding.

f. To block nonspecific protein interactions, flow in 1 $\times$ chamber volume of 1.5 mg/mL casein solution in PEM buffer and

incubate for 8 min.

g. Wash with PEM buffer to remove unbound casein.

h. Dilute 1 μL of microtubule (MT) filaments in 20 μL of warm taxol buffer and flow 15 μL of the diluted sample into the flow chamber.

i. Place the chamber inside the temperature-controlled enclosure of the microscope. Maintain the temperature at 37 $^{\circ}\text{C}$.

Note: Switch on the temperature control and set at 37 $^{\circ}\text{C}$ 1 h before the experiment for better results.

j. Check for microtubules at the surface of the chamber in the TRITC filter using a 60 $\times$ lens.

k. Add 1 $\times$ chamber volume of motility buffer and observe microtubule motility in the TRITC filter using a 60 $\times$ lens in an epifluorescence microscope.

l. Take images every 2 s for 2–5 min.

Note: To avoid drying of the chamber during imaging, wash the chamber with 15 μL of motility buffer after 20 min of imaging.

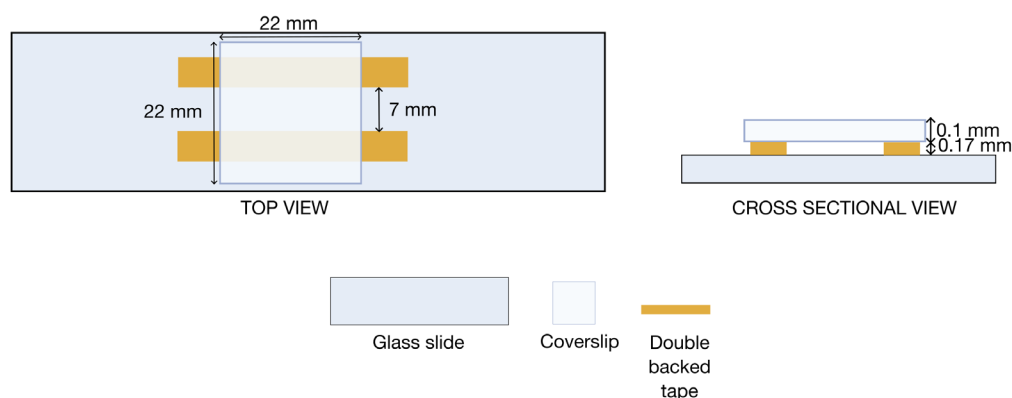


Figure 3. Schematic representing flow chambers used in motility assays. Representative schematics of the flow chamber setup used for motility assays. Double-backed tapes of 0.17 mm cross-sectional thickness were stuck on glass slides. Glass coverslips of dimensions 22 $\times$ 22 mm and 0.1 mm thickness were cleaned using acetone and KOH and adhered to the tape. This results in a flow chamber of volume $\sim$ 15 μL .

2. K401

a. Prepare flow chambers as done for K980 (D3.1).

b. Flow 1 $\times$ chamber volume of 1 mg/mL streptavidin in PEM buffer and incubate for 10 min.

c. Wash the chamber using PEM buffer.

d. Block the surface by incubating the flow chamber with 1 $\times$ chamber volume of 1.5 mg/mL casein solution for 8 min.

e. Wash out unbound casein using PEM buffer.

f. Add 15 μL of biotinylated kinesin (K401) to the streptavidin-coated coverslips.

g. Incubate for 20 min and wash unbound protein using PEM buffer.

h. Repeat steps D3.2f–g three times.

i. Add diluted microtubules and follow motility by introducing motility buffer as done for K980.

3. K910-980

a. Prepare flow chambers as done for K980 (section D3.1).

b. Flow 1 $\times$ chamber volume of 1 mg/mL purified anti-GFP nanobody and incubate for 10 min.

c. Wash the chamber using PEM buffer.

d. Block the surface by incubating the flow chamber with 1 $\times$ chamber volume of 1.5 mg/mL casein solution for 8 min.

e. Wash out unbound casein using PEM buffer.

f. Add 15 μL of K910-980 to the nanobody-coated coverslips.

g. Incubate for 20 min at 25 $^{\circ}\text{C}$ and wash unbound protein using PEM buffer.

h. Repeat steps D3.3f–g three times.

i. Add diluted microtubules and follow motility by introducing motility buffer as done for K980.

4. K401 and K910-980 mix
 - a. Prepare flow chambers as done previously.
 - b. Flow 1× chamber volume of 1 mg/mL streptavidin in PEM buffer and incubate for 10 min.
 - c. Wash the chamber using PEM buffer.
 - d. Flow 1× chamber volume of 1 mg/mL purified anti-GFP nanobody and incubate for 10 min.
 - e. Wash the chamber using PEM buffer.
 - f. Block the surface by incubating the flow chamber with 1× chamber volume of 1.5 mg/mL casein solution for 8 min.
 - g. Wash out unbound casein using PEM buffer.
 - h. For individual tail and motor ratios, add 15 µL of total protein concentration of 1 µM for each, as follows:

$C_{\text{Tail}}/C_{\text{Tail+Head}} = 0.25$: 0.75 µM of K401 and 0.25 µM of K910-980

$C_{\text{Tail}}/C_{\text{Tail+Head}} = 0.5$: 0.5 µM of K401 and 0.5 µM of K910-980

$C_{\text{Tail}}/C_{\text{Tail+Head}} = 0.75$: 0.25 µM of K401 and 0.75 µM of K910-980

- i. Incubate for 20 min at 25 °C and wash unbound protein using PEM buffer.
- j. Repeat steps D3.4f–g three times.
- k. Add 1× chamber volume of diluted microtubules and follow motility by introducing motility buffer as done for K980.

E. Quantification of microtubule gliding velocity

1. Background-subtract each gliding assay time series using the *background subtraction* menu in FIJI, followed by the *median filter* option (Sigma = 0.5).

Note: For better background correction, use a Gaussian filter with Sigma = 0.5.

2. Save the corrected file in .tif format.
3. Load each time series to the MATLAB window of FIESTA.
4. Add the time difference for the time series stack, in this case, 2,000 ms.
5. Enter the pixel size corresponding to the resolution; for the 60× lens used in this study, it is 106.1 nm.
6. Set the threshold for the time series. Correct if required.
7. Check the configuration of the image. Select *Only Filaments* option under tracking. Keep the remaining settings as default.
8. Select *Add stack to LOCAL queue* and then proceed with analyzing the stack.
9. Load the tracks as generated by FIESTA.
10. Measure instantaneous velocities from individual tracks for each filament using the 2D point-to-point velocity function in FIESTA.

F. Quantification of microtubule spatial patterns

1. Select motile microtubules for each representative field of view.
2. Manually segment the contour length, L (µm), and end-to-end distance, R (µm), using *Line Segment* tool in FIJI.
3. Mark each line segment as a separate ROI.
4. Overlay each image with the marked MT filaments.
5. Measure each distance using the *measure* tool in FIJI to extract values for both L and R .
6. Measure the bending factor, which is defined as the ratio of R/L , as follows:

Bending factor = R/L

G. Quantification of surface microtubule density

1. To measure microtubule surface density, prepare flow chambers as described for the gliding assay, and surface-immobilize both K401 and K910-980 as follows:

2. K401
 - a. Incubate the flow chambers with 15 µL of 1 mg/mL streptavidin for 10 min.
 - b. Wash unbound streptavidin with 15 µL of PEM buffer.

- c. Block the surface by incubating the flow chamber with 1 mg/mL casein solution.
- d. Incubate the flow chamber with 15 μ L of 1 μ M K401 for 20 min.
- e. Wash unbound K401 using PEM buffer.

3. K910-980

- a. Incubate the flow chambers with 15 μ L of 1 mg/mL anti-GFP nanobody for 10 min.
- b. Wash unbound nanobody with 15 μ L of PEM buffer.
- c. Block the surface by incubating the flow chamber with 1 mg/mL casein solution.
- d. Incubate the flow chamber with 15 μ L of 1 μ M K910-980 for 20 min.
- e. Wash unbound protein using PEM buffer.

4. For both proteins, flow in a diluted solution of microtubules as done for the gliding assay.

Note: For better comparability, try to use the same microtubule mix for both head and tail flow chambers.

5. Incubate the flow chambers with microtubules for 10 min.
6. Wash unbound microtubules using taxol buffer.
7. Image microtubules in the TRITC filter using the 100 $\times$ lens.
8. Background-correct the acquired images as described previously.
9. Manually count microtubules for representative fields of view.
10. Measure microtubule density by dividing the number of microtubules by the total image area (mm^2).

H. Microtubule motor co-localization assay

1. Assemble microtubules as described for the gliding assay.
2. Prepare flow chambers as done for the gliding assay.
3. Add 15 μ L of 100 μ g/mL of rabbit polyclonal anti-tetramethyl rhodamine antibody into the flow chamber.
4. Incubate for 15 min.
5. Wash excess antibody using PEM buffer.
6. Passivate the surface by incubating 1% Pluronic F127 for 20 min.
7. Wash the chamber with 15 μ L of PEM buffer.
8. Flow in microtubules and incubate for 10 min.
9. Add 15 μ L volumes of either 1 μ M of K560-GFP (head) or 1 μ M of GFP-K910-980 (tail).
10. Incubate for 15 min at 37 $^{\circ}$ C.
11. Wash out unbound protein by introducing 1 chamber volume of PEM buffer supplemented with 20 μ M paclitaxel.
12. Use the TRITC filter at 561 nm to observe rhodamine-labeled MTs and the FITC filter at 488 nm to observe the GFP-tagged proteins.
13. Merge the two channels using FIJI to look for co-localization.

Data analysis

All experiments should be performed with at least three independent measurements and for multiple fields of view. For statistical comparison across two groups, compare the mean of individual biological replicates across conditions using an unpaired two-tailed Student's t-test. $p > 0.05$ is considered non-significant (n.s.). For data analysis, *scipy.stats.ttest_ind* function in SciPy (v1.7.3) can be used in Python (v3.9.12).

Validation of protocol

This protocol has been used and validated in the following research article:

- Basu et al [8]. Spatial Coin-Tossing by Kinesin-1 Head and Tail Binding Collectively Drives Microtubule Patterns. *Molecular Biology of the Cell*.

Acknowledgments

Conceptualization, J.B. and C.A.A.; Investigation, J.B. and C.A.A.; Writing—Original Draft, J.B., K.S.; Writing—Review & Editing, A.J. and C.A.A.; Funding acquisition, C.A.A.; Supervision, C.A.A.

J. B. acknowledges IISER Pune for PhD studentship. K.S. acknowledges Dept. of Biotechnology, Govt. of India (DBTHRDPNU/JRF/BET-23/I/2023-2024/356) for PhD fellowship. The project was supported by intramural funds from IISER Pune to CAA.

Original research paper: Basu, J., Singh, K., Jannasch, A., & Athale, C. A. (2026) Spatial Coin-Tossing by Kinesin-1 Head and Tail Binding Collectively Drives Microtubule Patterns. *Molecular Biology of the Cell*. 37(3):ar25.

We are grateful to William Hancock for the full-length and rigor kinesin construct, K980 plasmid, and Jeff Gelles for the K401-BCCP plasmid. We thank Ron Vale for the gift of the K560 construct.

Competing interests

The authors declare no conflicts of interest.

Received: February 11, 2026; Accepted: April 14, 2026; Available online: May 12, 2026; Published: May 20, 2026

References

- Howard, J., Hudspeth, A. J. and Vale, R. D. (1989). Movement of microtubules by single kinesin molecules. *Nature*. 342(6246): 154–158. <https://doi.org/10.1038/342154a0>
- Chaphalkar, A. R., Jain, K., Gangan, M. S. and Athale, C. A. (2016). Automated Multi-Peak Tracking Kymography (AMTraK): A Tool to Quantify Sub-Cellular Dynamics with Sub-Pixel Accuracy. *PLoS One*. 11(12): e0167620. <https://doi.org/10.1371/journal.pone.0167620>
- Jain, K., Khetan, N. and Athale, C. A. (2019). Collective effects of yeast cytoplasmic dynein based microtubule transport. *Soft Matter*. 15(7): 1571–1581. <https://doi.org/10.1039/c8sm01434e>
- Bieling, P., Telley, I. A., Piehler, J. and Surrey, T. (2008). Processive kinesins require loose mechanical coupling for efficient collective motility. *EMBO Rep*. 9(11): 1121–1127. <https://doi.org/10.1038/embor.2008.169>
- Wang, Z., Zhang, F., He, J., Wu, P., Tay, L. W. R., Cai, M., Nian, W., Weng, Y., Qin, L., Chang, J. T., et al. (2017). Binding of PLD2-Generated Phosphatidic Acid to KIF5B Promotes MT1-MMP Surface Trafficking and Lung Metastasis of Mouse Breast Cancer Cells. *Dev Cell*. 43(2): 186–197.e7. <https://doi.org/10.1016/j.devcel.2017.09.012>
- Seeger, M. A. and Rice, S. E. (2010). Microtubule-associated Protein-like Binding of the Kinesin-1 Tail to Microtubules. *J Biol Chem*. 285(11): 8155–8162. <https://doi.org/10.1074/jbc.m109.068247>
- Lu, W., Winding, M., Lakonishok, M., Wildonger, J. and Gelfand, V. I. (2016). Microtubule–microtubule sliding by kinesin-1 is essential for normal cytoplasmic streaming in *Drosophila* oocytes. *Proc Natl Acad Sci USA*. 113(34): e1522424113. <https://doi.org/10.1073/pnas.1522424113>
- Basu, J., Singh, K., Jannasch, A. and Athale, C. A. (2026). Spatial coin-tossing by kinesin-1 head and tail binding collectively drives microtubule patterns. *Mol Biol Cell*. 37(3): ee25–09–0427. <https://doi.org/10.1091/mbc.e25-09-0427>
- Bicek, A. D., Tüzel, E., Demtchouk, A., Uppalapati, M., Hancock, W. O., Kroll, D. M. and Odde, D. J. (2009). Anterograde Microtubule Transport Drives Microtubule Bending in LLC-PK1 Epithelial Cells. *Mol Biol Cell*. 20(12): 2943–2953. <https://doi.org/10.1091/mbc.e08-09-0909>
- Gittes, F., Meyhöfer, E., Baek, S. and Howard, J. (1996). Directional loading of the kinesin motor molecule as it buckles a microtubule. *Biophys J*. 70(1): 418–429. [https://doi.org/10.1016/s0006-3495\(96\)79585-1](https://doi.org/10.1016/s0006-3495(96)79585-1)
- Vilfan, A., Subramani, S., Bodenschatz, E., Golestanian, R. and Guido, I. (2019). Flagella-like Beating of a Single Microtubule. *Nano Lett*. 19(5): 3359–3363. <https://doi.org/10.1021/acs.nanolett.9b01091>
- Bourdieu, L., Duke, T., Elowitz, M. B., Winkelmann, D. A., Leibler, S. and Libchaber, A. (1995). Spiral Defects in Motility Assays: A Measure of Motor Protein Force. *Phys Rev Lett*. 75(1): 176–179. <https://doi.org/10.1103/physrevlett.75.176>
- Gittes, F., Mickey, B., Nettleton, J. and Howard, J. (1993). Flexural rigidity of microtubules and actin filaments

- measured from thermal fluctuations in shape.. *J Cell Biol.* 120(4): 923–934. <https://doi.org/10.1083/jcb.120.4.923>
14. Brangwynne, C. P., MacKintosh, F. C. and Weitz, D. A. (2007). Force fluctuations and polymerization dynamics of intracellular microtubules. *Proc Natl Acad Sci USA.* 104(41): 16128–16133. <https://doi.org/10.1073/pnas.0703094104>
 15. Subramanian, R. and Gelles, J. (2007). Two Distinct Modes of Processive Kinesin Movement in Mixtures of ATP and AMP-PNP. *J Gen Physiol.* 130(5): 445–455. <https://doi.org/10.1085/jgp.200709866>
 16. Hancock, W. O. and Howard, J. (1998). Processivity of the Motor Protein Kinesin Requires Two Heads. *J Cell Biol.* 140(6): 1395–1405. <https://doi.org/10.1083/jcb.140.6.1395>
 17. Katoh, Y., Nozaki, S., Hartanto, D., Miyano, R. and Nakayama, K. (2015). Architectures of multisubunit complexes revealed by a visible immunoprecipitation assay using fluorescent fusion proteins. *J Cell Sci.* 128(12): 2351–2362. <https://doi.org/10.1242/jcs.168740>
 18. Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L. and Mitchison, T. (1991). [39] Preparation of modified tubulins. *Meth Enzymol.* 196: 478–485. [https://doi.org/10.1016/0076-6879\(91\)96041-o](https://doi.org/10.1016/0076-6879(91)96041-o)
 19. Gibson, D. G., Young, L., Chuang, R. Y., Venter, J. C., Hutchison, C. A. and Smith, H. O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat Methods.* 6(5): 343–345. <https://doi.org/10.1038/nmeth.1318>
 20. Castoldi, M. and Popov, A. V. (2003). Purification of brain tubulin through two cycles of polymerization–depolymerization in a high-molarity buffer. *Protein Expression Purif.* 32(1): 83–88. [https://doi.org/10.1016/s1046-5928\(03\)00218-3](https://doi.org/10.1016/s1046-5928(03)00218-3)
 21. Basu, J., Soni, A. and Athale, C. A. (2025). Physical effects of crowdant size and concentration on collective microtubule polymerization. *Biophys J.* 124(5): 789–806. <https://doi.org/10.1016/j.bpj.2025.01.020>
 22. Jain, K., Basu, J., Roy, M., Yadav, J., Patil, S. and Athale, C. A. (2021). Polymerization kinetics of tubulin from mung seedlings modeled as a competition between nucleation and GTP-hydrolysis rates. *Cytoskeleton.* 78(9): 436–447. <https://doi.org/10.1002/cm.21694>
 23. Woehlke, G., Ruby, A. K., Hart, C. L., Ly, B., Hom-Booher, N. and Vale, R. D. (1997). Microtubule interaction site of the kinesin motor. *Cell.* 90(2): 207–216. <https://doi.org/10.1038/35036069>