Membrane-bound cargos in cells are generally transported by multiple kinesin motors. Quantifying the bimolecular on-rate of motors for their microtubule track is important for understanding of multi-motor transport but is complicated by diffusion of the motors in the plane of the lipid bilayer. Here, we describe a method to measure the kinesin on-rate that uses a modified microtubule gliding assay performed on a supported lipid bilayer and detects motor binding by a local increase in fluorescence.
Measuring microtubule binding kinetics of membrane-bound kinesin motors using supported lipid bilayers

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SUMMARY
Membrane-bound cargos in cells are generally transported by multiple kinesin motors. Quantifying the bimolecular on-rate of motors for their microtubule track is important for understanding of multi-motor transport but is complicated by diffusion of the motors in the plane of the lipid bilayer. Here, we describe a method to measure the kinesin on-rate that uses a modified microtubule gliding assay performed on a supported lipid bilayer and detects motor binding by a local increase in fluorescence.
For complete details on the use and execution of this protocol, please refer to Jiang et al. (2019).

BEFORE YOU BEGIN
The protocol below describes how to quantify the microtubule binding kinetics of GFP-tagged kinesin-1 motors (K560GFP-AviC) bound to a supported lipid bilayer (SLB) by measuring the rate of local fluorescence increase upon the GFP-tagged motors binding to a newly landed microtubule (Figure 1). This protocol can in principle be applied to any motor (kinesin, dynein, myosin) that moves along a cytoskeletal filament.

Preparation of multilamellar vesicles (MLVs)

Θ Timing: 6 h

In this step, lipids are mixed in a desired molar ratio in chloroform. Evaporation of chloroform leaves a lipid film on the glass vessel. Upon rehydration with buffer, the lipids swell and form onion-like multilamellar vesicles. The subsequent freeze-thaw cycles result in repeated vesicle fracture, which reduces both the lamellarity and size of the vesicles.

1. Mix POPC, DSPE-PEG(2000) Biotin, and Atto 647N DOPE in 500 μL chloroform (total lipid concentration 5 mg/mL). DSPE-PEG(2000) Biotin is added at 0.1–0.6 mol% to control motor density on the membrane. Atto 647N DOPE is added at 0.05 mol% for visualization of the lipid bilayer. If desired, up to 30 mol% cholesterol can be incorporated to reduce the diffusivity of the membrane without causing phase separation. See Figure 2 for example traces of fluorescence recovery after photobleaching (FRAP) measurements carried out on SLBs with or without cholesterol.

△ CRITICAL: A linker of sufficient length between the biotin and the lipid head group is critical to relieve the steric hinderance for NeutrAvidin binding to the lipid bilayer.
DSPE-PEG(2000) Biotin, which has \((\text{OCH}_2\text{CH}_2)_{45}\) as the linker between the biotin and the lipid head group, significantly improves the surface coverage of NeutrAvidin compared to 18:1 biotinyl PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(biotinyl), no linker) or 18:1 biotinyl Cap PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(cap biotinyl), \((\text{CH}_2)_5\) as linker).

**Note:** We found that changing the fraction of DSPE-PEG(2000) Biotin in the lipid bilayer and incubating it with saturating level of NeutrAvidin-bound biotinylated motors is a reliable and consistent way to change motor coverage at high densities. If extremely low motor densities are desired, they can be achieved by incubating substoichiometric level of motors with lipid bilayers containing 0.1 mol\% DSPE-PEG(2000) Biotin. We do not recommend tuning motor density by reducing DSPE-PEG(2000) Biotin to below 0.1 mol\% because: 1) it is difficult to accurately measure such a small quantity of lipids; 2) strong hydration of PEG(2000) modifies the properties of the lipid bilayer. Thus, the amount of DSPE-PEG(2000) Biotin should be tuned in a narrow range to avoid significant changes in the properties of the SLB.

**Note:** Atto 647N is very bright and we found that 0.05 mol\% works well for routine check of SLB homogeneity using a total internal reflection fluorescence (TIRF) microscope. However, such a low concentration does not provide sufficient signal-to-noise ratio for fluorescence recovery after photobleaching (FRAP) measurement, in which case 0.5 mol\% is needed.

**Note:** Make sure to use glass containers, pipettes and syringes when handling chloroform. This should also be done in a fume hood.

2. Evaporate chloroform in a rotary evaporator at 450 mmHg pressure, 260 rpm. Once the bulk chloroform is evaporated (~1.5 h for 500 μL), leave the lipids in the evaporator for another 3 h to remove any trace chloroform.

**Note:** If a rotary evaporator is not available, the bulk chloroform can be dried under a steady stream of nitrogen gas. Then leave the lipid film under vacuum for 3 h.

3. Rehydrate the lipids at 0.5 mg/mL with 5 mL BRB80 at 20°C–24°C for 30 min. Vortex periodically to make a uniform lipid suspension. The lipid suspension will look cloudy at this stage.
4. Subject the lipid suspension to 10 freeze-thaw cycles by alternately placing it in liquid nitrogen and a warm water bath (~40°C). It should appear less cloudy (but not clear) as the lamellarity and the size of the vesicles decrease.

**Pause point:** Prior to the last freeze-thaw cycle, the lipids can be aliquoted (1 ml each), flash frozen in liquid nitrogen and stored at ~80°C. The MLVs should remain viable for 1–2 months. We avoid using MLVs older than 2 months due to concerns of lipid oxidation.

**Preparation of small unilamellar vesicles (SUVs)**

- **Timing:** 1 h

In this step, SUVs are formed by passing the MLVs through membrane filters with small pore size.

5. Thaw a MLV aliquot.

6. Assemble the mini-extruder as instructed by Avanti (https://avantilipids.com/divisions/equipment-products/mini-extruder-assembly-instructions).

7. Extrude lipids 11 times through one 100 nm membrane filter, followed by 21 times through one 30 nm membrane filter (See instructions from Avanti https://avantilipids.com/divisions/equipment-products/mini-extruder-extrusion-technique). For the lipid compositions used in this protocol, this method produces SUVs with an average diameter of ~70 nm when measured by dynamic light scattering (DLS) (Figure 3).

**Note:** Significant force is needed to extrude the lipids through the 30 nm membrane filter. Change the membrane filter if extrusion becomes too difficult.

**Pause point:** The SUVs can be stored at 4°C for 1–2 weeks.

**Preparing PDMS and cleaning coverslips**

- **Timing:** 1 day
Polydimethylsiloxane (PDMS) is cured at 20°C–24°C for 24 h. Cured PDMS will be cut into spacers that are placed on the coverslips to make sample wells in which SLBs are formed (Figure 4). The coverslips are first cleaned in hot 7x cleaning solution to remove the dirt and expose the intrinsically hydrophilic glass surface. They are then annealed at high temperature to make the glass surface extremely smooth.

8. Polymerize PDMS by thoroughly mixing 5 g base and 0.5 g curing agent from the Silicone Elastomer Kit. Pour the mixture into a 100 x 15 mm disposable petri dish. Degas the mixture under vacuum. Leave the petri dish on a level surface and allow PDMS to cure for 24 h.

9. Dilute 7x cleaning solution in ddH2O (1:7 dilution) in a large glass dish. Submerge coverslips (held on ceramic racks) in the cleaning solution. The solution looks cloudy at 20°C, it will turn clear when heated.

10. Place the dish on a hot plate set to 5 to 6 (on a scale of 1 to 10). The solution should just turn clear but not boiling. Adjust the hot plate temperature if needed. Maintain the heat for 2 h. Keep an eye on the water level, add more water if needed to keep the coverslips submerged.

11. Rinse coverslips thoroughly under running ddH2O. The coverslips should become very hydrophilic at this stage and water should easily cover the entire coverslip surface during rinsing.

12. Blow dry the coverslips with clean nitrogen gas.

13. Transfer the coverslips to the kiln. Program the kiln to hold temperature at 550°C for 6 h. The coverslips can stay in the kiln overnight if the program ends after work hours.

14. Collect the coverslips after annealing. To keep them clean, we place the coverslip box inside a larger clean box and keep them in a drawer.

**Pause point:** Hydrophilic surfaces easily collect dirt so we recommend using the coverslips within 1–2 weeks after annealing. The petri dish containing cured PDMS can be stored in a drawer. PDMS is an inert material and it can be used for experiments as long as it is clean.

**Purification of active kinesin motors by microtubule pelleting assay**

© Timing: 1.5 h

In this step, the biotinylated kinesin motors are first incubated with excess NeutrAvidin. Motors are then allowed to bind to microtubules in the presence of AMPPNP, a non-hydrolyzable ATP analog. Motors that are capable of binding will co-pellet with microtubules. The microtubule pellet is then resuspended in ATP to release the active motors. A second spin separates the active motors in the supernatant from the ones that irreversibly bind to microtubules in the pellet.
15. Prepare solutions to be used in the pelleting assay. Each of our airfuge tubes can hold ~300 μL liquid when completely filled. We fill 200 μL reaction mixture in each tube, which is enough for 3 assays (55 μL for each assay). The recipes below give enough solutions for 3 reaction mixtures. Keep the solutions at 20°C–24°C.

a. 1 mg/mL BSA: 980 μL BRB80 + 20 μL BSA (50 mg/mL)

b. 10 μM taxol: 495 μL BRB80 + 5 μL taxol (1 mM)

c. Pelleting buffer (10 μM taxol, 100 μM MgAMPPNP, 1 mM DTT): 987 μL BRB80 + 10 μL taxol (1 mM) + 2 μL MgAMPPNP (50 mM) + 1 μL DTT (1 M)

d. Resuspension buffer (1 μM taxol, 2 mM MgATP, 1 mM DTT, 20 mM D-glucose): 968 μL BRB80 + 1 μL taxol (1 mM) + 20 μL MgATP (100 mM) + 1 μL DTT (1 M) + 10 μL D-glucose (2 M)

Note: Briefly vortex the solution immediately after adding taxol to BRB80 so that DMSO quickly disperses in the buffer, to avoid taxol precipitation at the DMSO-water interface.

16. Thaw 3 tubulin (40 μM) aliquots. Add 0.6 μL DMSO, 0.5 μL MgCl2 (100 mM), 0.5 μL MgGTP (25 mM) to each aliquot and mix well. Polymerize microtubules by incubating in a 37°C water bath for 30 min. Proceed with the following steps while the microtubules are polymerizing.

17. Fill 3 airfuge tubes with 1 mg/mL BSA to the top to block the surface. Incubate for 10 min at 20°C–24°C. Then rinse each tube 3 times with BRB80.

18. During the 10 min incubation with BSA, in a separate airfuge tube, add 8 μM NeutrAvidin (96 μL NeutrAvidin (16.7 μM) + 104 μL pelleting buffer), spin at 30 psi for 5 min to remove any aggregates.

19. Collect the supernatant of NeutrAvidin. In each airfuge tube blocked with BSA, add 2 μM NeutrAvidin (50 μL supernatant), 200 nM K560GFP-AviC, and pelleting buffer (145 μL minus the volume of K560GFP-AviC). Incubate at 20°C–24°C for 10 min.

Note: We noted that if we first loaded NeutrAvidin to the SLB surface, microtubules bound non-specifically to the NeutrAvidin in the absence of kinesin motors. To overcome this problem, we first incubate NeutrAvidin with kinesin motors before purifying the active motors. Any free NeutrAvidin will be removed after the microtubule pelleting assay. Therefore, in the final assay system, there will be no NeutrAvidin that is not occupied by a kinesin motor. This eliminates any non-specific interaction with the microtubule that may interfere with the measurement of motor binding kinetics.

Note: NeutrAvidin has 4 binding sites for biotin, thus the 10× excess NeutrAvidin is necessary to avoid cross-linking of motors.

20. The microtubules should finish polymerizing at this point.
a. To one aliquot, add 490 μL taxol solution (10 μM) made in step 15b to stabilize the microtubules. Shear the microtubules by passing the solution twice through a 30 gauge needle (at a flow rate ~100 μL/s). These microtubules are kept at 20°C–24°C, and will be used for motor accumulation assay later.

Note: We prefer to use relatively short microtubules for the accumulation assay. For microtubules longer than 10 μm, one end of the microtubule may land on the surface at an earlier time than the other end, which results in a biphasic rise in motor accumulation signal.

b. The other two aliquots will be used for pelleting assay. To each reaction mixture made in step 19, add 5 μL polymerized microtubules (final concentration 1 μM) and incubate at 20°C–24°C for 5 min. The microtubules are stabilized by taxol present in the pelleting buffer.

21. Spin at 20 psi for 3 min.

Note: A gentle spin at this step makes it easier to resuspend the microtubule pellet.

22. Discard the supernatant containing inactive motors that do not bind the microtubules, along with excess NeutrAvidin. Resuspend each microtubule pellet with 200 μL resuspension buffer. Incubate at 20°C–24°C for 10 min.

23. Spin at 30 psi for 5 min.

24. Collect the supernatant containing active motors with NeutrAvidin bound. The active motors can be kept on ice for several hours. Discard the microtubule pellet that contains inactive motors that irreversibly bind microtubules.

25. This protocol typically yields 50–100 nM active motors. The concentration can be measured using a spectrofluorometer or a plate reader. A calibration curve of GFP intensity versus motor concentration is obtained by measuring the fluorescence signal of 0–200 nM KS60GFP-AviC diluted in resuspension buffer (this volume is not accounted for in step 15d). The concentration of purified motor-NeutrAvidin complex is obtained by comparing its fluorescence signal to the calibration curve.

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| K560GFP-AviC        | Jiang et al., 2019 | N/A |
| Bovine brain tubulin| Jiang et al., 2019 | N/A |
| NeutrAvidin         | Thermo Fisher | Cat#: 31000 |
| 1-Palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC) | Avanti Polar Lipids | Cat#: 850457 |
| 1,2-Distearoyl-sn-glycero-3-phosphoethanolamine-N-[biotin(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG(2000) Biotin) | Avanti Polar Lipids | Cat#: 880129 |
| Atto 647N DOPE      | Sigma | Cat#: 42247-1MG |
| 1,4-Piperazinediethanesulfonic acid (PIPES) | Sigma | Cat#: P6757 |
| Ethylene glycol-bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) | Millipore | Cat#: 4100 |
| Magnesium Chloride (MgCl$_2$, 2M) | Quality Biological | Cat#: 340-034-721 |
| Adenosine 5'-triphosphate disodium salt hydrate (ATP) | Sigma | Cat#: A2383-25G |
| Adenosine-5'-[(3H,7H)-(3R,7R)-imidazo)]triphosphate, tetralithium salt (AMPPNP) | Jena Bioscience | Cat#: NU-407-50 |
| Guanosine 5'-triphosphate, disodium salt tritylphosphate (GTP) | Jena Bioscience | Cat#: NU-1012-10G |
| Paclitaxel (taxol)  | Sigma | Cat#: T7191-5MG |
| Dimethyl sulfoxide (DMSO) | VWR | Cat#: N182-5X10ML |
| BSA, Fraction V     | Millipore | Cat#: 2930-100GM |
| Dextrose (D-glucose) | Sigma | Cat#: DX0145-1 |

(Continued on next page)
**Continued**

### MATERIALS AND EQUIPMENT

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Glucose oxidase, Aspergillus | Sigma | Cat#: 345386-10KU |
| Catalase from bovine liver | Sigma | Cat#: C1345-1G |
| Dithiothreitol (DTT) | Thermo Scientific | Cat#: 20290 |
| 7X Cleaning Solution | MP Biomedicals | Cat#: 097667093 |
| SYLGARD™ 184 Silicone Elastomer Kit | Electron Microscopy Sciences | Cat#: 24236-10 |

**Software and algorithms**

| Fiji | https://imagej.net/Fiji | N/A |
| MATLAB | MathWorks | N/A |

**Other**

| Self-Cleaning Dry Vacuum System | Welch | 2025 |
| Rotary evaporator | Heidolph | Collegiate |
| Glassware set for rotary evaporator | Heidolph | G1 |
| Mini-extruder | Avanti Polar Lipids | Cat#: 610000 |
| Filter Support | Avanti Polar Lipids | Cat#: 610014 |
| Polycarbonate Membranes 0.1 μm | Avanti Polar Lipids | Cat#: 610005 |
| Polycarbonate Membranes 0.03 μm | Avanti Polar Lipids | Cat#: 610002 |
| Coverslips, #1.5, 24 x 30 mm | Corning | Cat#: 2980-243 |
| Klin | Paragon | SC2 |
| Plasma Cleaner | Harrick Plasma | PDC-32G |
| Biopsy punch (6 mm) | Acuderm | Cat#: P625 |
| 30-gauge needle | Hamilton | Cat#: 90030 |
| Airfuge | Beckman Coulter | Cat#: 350624 |
| Total internal reflection fluorescence (TIRF) microscope | Nikon | TE2000 |

### BRB80, pH 6.9

| Reagent | Final concentration | Amount |
|---------|---------------------|--------|
| KOH     | n/a                 | 5.39 g  |
| PIPES   | 80 mM               | 24.19 g |
| EGTA    | 1 mM                | 0.38 g  |
| MgCl₂ (2 M) | 1 mM             | 0.5 mL  |
| ddH₂O   | n/a                 | To 1 L  |
| Total   | n/a                 | 1 L     |

The KOH amount listed in the table is ~80% of the total amount needed. Add KOH pellet first to raise the pH and allow PIPES to dissolve. After adding all components in the table, use KOH (10 M) to adjust the pH to 6.9. Filter the buffer through a 0.2 μm filter. The buffer can be stored at 20°C–24°C for at least a year.

**Biotinylated and fluorescently tagged kinesin motors**

We use a truncated kinesin-1 motor dual labeled with GFP and an AviTag (K560GFP-AviC). The protein is bacterially expressed and purified by affinity chromatography through its 6x His tag. The protocol was described in (Uppalapati et al., 2009). The proteins in Ni column elution buffer are supplemented with 10 μM ATP, 4 mM DTT, and 10% sucrose, aliquoted, flash frozen, and stored at −80°C. The shelf life varies with motor species and buffer compositions, and needs to be determined empirically. We have not noticed any changes in motor activity of the construct used in this study after 6 months to a year.

**40 μM Bovine brain tubulin**

For detailed protocol, see (Uppalapati et al., 2009). Tubulin is flash frozen as 10 μL aliquots. They can be kept at −80°C for at least two years.
16.7 μM NeutrAvidin in BRB80
Make 10 mg/mL NeutrAvidin by adding 1 mL of 10% glycerol to dissolve all the NeutrAvidin (10 mg) in a bottle. Then add 9 mL BRB80 to dilute it to 1 mg/mL (16.7 μM). Make 25 μL aliquots and flash freeze. They can be stored at −80°C for at least a year.

50 mg/mL BSA in BRB80
Use 20 mL BRB80 to dissolve 2 g BSA (100 mg/mL). Filter through a 0.45 μm filter. Measure concentration by absorbance at 280 nm (extinction coefficient 0.66 (mg/mL)⁻¹ cm⁻¹). Use BRB80 to adjust the concentration to 50 mg/mL. Make 1 mL (for re-aliquoting) and 25 μL aliquots (for single use in experiments) and flash freeze. BSA can be stored at −80°C for at least a year.

1 mM Taxol
Use 5.8 mL DMSO to dissolve all the Paclitaxel (5 mg) in the bottle. Make 1 mL and 25 μL aliquots and store at −20°C. The aliquots remain good for at least 6 months to a year, as long as water does not accumulate in the aliquots.

△ CRITICAL: Anhydrous DMSO has a freezing point around 18°C, however, the freezing point of DMSO/water mixture drops as the water content increases (then it increases again as water becomes dominant) (Havemeyer, 1966). Occasionally we notice that the taxol aliquots appear aqueous in the −20°C freezer (and this typically starts from the top of the aliquot). We attribute this to the hygroscopic properties of DMSO which may be exacerbated by temperature fluctuations when the freezer is accessed frequently. Taxol is extremely insoluble in water so the aliquots should be discarded if significant water has accumulated. We recommend making 25 μL instead of 10 μL aliquots so they can sustain temperature fluctuations better, as well as storing taxol aliquots in a less accessed region in the freezer.

1 M DTT
Dissolve 308.5 mg DTT in 2 mL ddH2O. Filter through a 0.2 μm filter. Make 10 μL aliquots and store at −20°C. Due to concerns of oxidation, we recommend remaking DTT stock solutions every 6 months to a year.

100 mM MgATP
Dissolve 551.14 mg ATP in 9.5 mL ddH2O. Add 500 μL MgCl₂ (2 M) and mix well. Filter through a 0.2 μm filter. Make 1 mL and 25 μL aliquots. They can be stored at −20°C for at least a year.

50 mM MgAMPPNP
Use 1.95 mL ddH₂O to dissolve all the AMPPNP (50 mg) in the bottle. Add 50 μL MgCl₂ (2 M) and mix well. Filter through a 0.2 μm filter. Make 25 μL aliquots. They can be stored at −20°C for at least a year.

25 mM MgGTP
Dissolve 65 mg GTP in 5 mL ddH₂O. Add 62.5 μL MgCl₂ (2 M) and mix well. Filter through a 0.2 μm filter. Make 500 μL and 5 μL aliquots. They can be stored at −20°C for at least a year.

100 mM MgCl₂
Dilute 250 μL MgCl₂ (2 M) with 4.75 mL ddH₂O. Filter through a 0.2 μm filter. Make 500 μL and 5 μL aliquots. They can be stored at −20°C for at least a year.

DMSO
DMSO used for microtubule polymerization is stored at −20°C as 5 μL aliquots. The aliquots remain good as long as water does not accumulate in them.
**2 M D-glucose**
Dissolve 3.6 g Dextrose in ddH₂O (total volume 10 mL). Filter through a 0.2 μm filter. Make 1 mL and 25 μL aliquots, store at −20°C. The aliquots remain good for at least two years.

**2 mg/mL glucose oxidase**
Dissolve the whole bottle of glucose oxidase (10 KU, 250 U/mg) in 20 mL cold BRB80. Make 500 μL and 10 μL aliquots and flash freeze. They can be stored at −80°C for at least a year.

**0.8 mg/mL catalase**
Dissolve 16 mg catalase in 20 mL cold BRB80. Make 500 μL and 10 μL aliquots and flash freeze. They can be stored at −80°C for at least a year.

**STEP-BY-STEP METHOD DETAILS**

**Perform motor accumulation assay**

© **Timing:** 40 min per sample

Kinesin-NeutrAvidin complexes purified in the previous step are loaded to a freshly formed SLB, and motor accumulation upon addition of microtubules is visualized using TIRF microscopy.

1. Make solutions for the accumulation assay. The recipes listed below are enough for 3 assays. Keep the solutions at 20°C–24°C.
   a. If performing accumulation assay in ATP:
      i. Motor buffer (2 mM ATP, 1 mM DTT, 20 mM D-glucose): 969 μL BRB80 + 20 μL MgATP (100 mM) + 1 μL DTT (1 M) + 10 μL D-glucose (2 M)
      ii. Motility buffer (1 μM taxol, 2 mM ATP, 1 mM DTT, 40 mM D-glucose): 958 μL BRB80 + 1 μL taxol (1 mM) + 20 μL MgATP (100 mM) + 1 μL DTT (1 M) + 20 μL D-glucose (2 M)
   b. If performing accumulation assay in AMPPNP:
      i. Motor buffer (1 mM DTT, 20 mM D-glucose): 989 μL BRB80 + 1 μL DTT (1 M) + 10 μL D-glucose (2 M)
      ii. Motility buffer (1 μM taxol, 2 mM AMPPNP, 1 mM DTT, 40 mM D-glucose): 938 μL BRB80 + 1 μL taxol (1 mM) + 40 μL MgAMPPNP (50 mM) + 1 μL DTT (1 M) + 20 μL D-glucose (2 M)
2. Use a plasma cleaner to treat one of the annealed coverslips on high RF level for 5 min. Meanwhile, allow the SUVs to come to 20°C–24°C.

**Note:** The coverslips cleaned in 7x cleaning solution and annealed at high temperature already have hydrophilic and smooth surfaces. However, since dirt can easily accumulate on hydrophilic surfaces, we plasma clean the coverslip right before forming the SLB to ensure the hydrophilicity of the surface.

**Note:** Allowing the cold solution to first equilibrate to room temperature avoids introducing air bubbles, which form as the solution temperature rises, to the SLB.

3. Cut a small piece of PDMS (~1 cm x 1 cm, ~1 mm thick) and use a 6 mm biopsy punch to cut a well in the center. Remove dust from the PDMS spacer by a piece of tape. Place the spacer on a plasma treated coverslip. A spacer free of dust should adhere to the coverslip by itself, which forms a sample well (Figure 4A).

**Δ CRITICAL:** It is critical to use an inert material, such as PDMS, as the spacer. Double-sided tape is commonly used as a spacer between a coverslip and a glass slide to form a flow cell. However, the extensive rinsing of the chamber with buffers in this assay results in dissolution of the adhesives on the tape into the sample chamber, causing defects in the SLB. For example, high concentration of detergent can cause the formation of membrane tubules.
4. Add 50 μL of SUVs to the well (Figure 4B) and incubate for 10 min. Meanwhile, transfer 55 μL active kinesin-NeutrAvidin mixture into a separate tube and allow it to equilibrate to 20°C–24°C.

5. Wash with 500 μL BRB80 in steps of 50 μL to remove excess SUVs. For each wash, add 50 μL BRB80 to the well, mix the solution by gently pipetting ~5 times, then remove 50 μL solution from the well. There should always be 50 μL solution left in the well to prevent expose of the SLB to the air.

6. Wash the SLB with 300 μL motor buffer in steps of 50 μL.

7. Add 50 μL of active kinesin-NeutrAvidin complexes that have come to 20°C–24°C. Gently pipet a few times to mix. Incubate for 10 min.

8. Wash with 250 μL motility buffer in steps of 50 μL.

9. Immediately before imaging, add 50 μL motility buffer to the sample well to make a total volume of 100 μL. Add 1 μL of glucose oxidase and 1 μL of catalase (final concentration 0.02 mg/mL and 0.008 mg/mL, respectively) to the well, use a p100/p200 pipet (set to 50 μL) to gently mix the solution.

10. Check SLB quality by Atto 647N fluorescence and motor signal homogeneity by GFP fluorescence using a total internal reflection fluorescence (TIRF) microscope. Troubleshooting 1

11. Take snapshots of the GFP signal in 5 different regions. These snapshots will be used for motor density calculation later (see steps 14–17).

△ CRITICAL: Since only accumulation rate but not steady-state fluorescence intensity is needed for calculating the $k_{on}$ and $k_{off}$ (Figure 8), it is not necessary to use the same frame rate for accumulation assays (step 12) carried out at different motor densities. However, the same exposure time must be used for capturing the snapshots to be used for motor density calculation.

12. Move the sample to a new region. When the sample is in focus, add microtubules made in step 20a under the ‘Before you begin’ section (final concentration 16–32 nM), pipet quickly and gently to mix, then immediately start taking a video in the GFP channel. Once a video is finished, quickly find another region of interest and start taking the next video. We usually take 3 videos per sample. The frame rate and movie duration are chosen according to the binding kinetics. For kinesin-1 motors, we take 100-s videos at 10 fps. Troubleshooting 2

**Note:** The success of this assay relies on capturing as many newly landed microtubules as possible during the course of the video. It is best to first find a good field of view under the microscope, then add the microtubules and immediately start recording. The frequency of microtubule landing events drops very quickly, so only the first few videos contain information for analysis. Additionally, as more microtubules become engaged with motors, motor depletion may become a concern and free motor density can no longer be assumed constant. A microscope equipped with a drift correction system is helpful but not necessary for the success of the experiment. Our microscope is not equipped with a drift correction system, and we tape the coverslip to the stage to keep it stable. The thin PDMS spacer allows us to hold the pipette at an angle when adding and mixing solution in the sample well and seeing it from the side, both of which help to prevent drift caused by sample addition on the stage.

**Note:** The final microtubule concentration needs to be adjusted based on the binding kinetics. A proper microtubule concentration should allow sufficient landing events to occur at the beginning of the assay, but prevent microtubule overlapping. This concentration range can be determined by pilot experiments where the density of gliding microtubules at steady state is observed at two extreme motor densities when varying amount of microtubules is added.

**Note:** The correct imaging parameters need to be explored in trial experiments. The exposure time should be long enough that motor accumulation signal along microtubules at steady
state is significantly higher than the background signal, and it needs to be short enough that \( \geq 50 \) frames are captured before accumulation reaches steady state.

13. Repeat accumulation assay at different motor densities. See step 14 for instructions on controlling motor density.

Quantify motor density

© Timing: 40 min per sample

Motor density can only be directly counted at extremely low densities; however, to achieve sufficient motor accumulation signal, most assays need to be carried out at high motor densities where direct counting is not feasible. Therefore, we first ensure that the mean GFP fluorescence scales linearly with surface motor density, and then directly count motor number at low density regimes and extrapolate to higher motor densities.

14. Follow steps 1–10 to prepare samples. Motor densities on the SLBs can be controlled as follows:
   a. At high motor densities, a saturating amount of kinesin-NeutrAvidin complexes (as in step 7) is added to the SLB, and motor density is controlled by the fraction of DSPE-PEG(2000) Biotin (0.1%–0.6%) in the SLB.
   b. At low motor densities, SLBs containing 0.1% DSPE-PEG(2000) Biotin are incubated with kinesin-free NeutrAvidin spiked with varying amounts of kinesin-NeutrAvidin complexes (total concentration of kinesin-free NeutrAvidin and kinesin-NeutrAvidin is 50 nM), so that only a fraction of DSPE-PEG(2000) Biotin is occupied by kinesin motors. The desired low motor density range for accumulation assay depends on the biochemical properties of the motor. For kinesin-1 motors, we were able to observe motor accumulation when at least 5 nM kinesin-NeutrAvidin was added.

15. For each sample, take snapshots of the GFP signal in 5 different regions and calculate the mean intensity. Plot GFP intensity against the equivalent biotin concentration in the SLB (DSPE-PEG(2000) Biotin (%) \times \frac{\text{kinesin-NeutrAvidin}}{50 \text{ nM}}). If there is a linear relationship, it is valid to use the extrapolation method. Proceed with motor counting in the next step. Trouble-shooting 3

16. Count the density of diffusing kinesin motors when individual molecules can be visualized. Under our conditions, we use 1/150 to 1/50 spiking ratios of kinesin-NeutrAvidin (0.3–1 nM) to free NeutrAvidin on SLBs containing 0.1% DSPE-PEG(2000) Biotin.

17. Based on the counting result, extrapolate the numbers to high motor densities.

EXPECTED OUTCOMES

The GFP signal from kinesin should appear homogeneous prior to microtubule landing, and a local increase in the GFP signal should appear once a microtubule lands (Methods video S1). Microtubules should glide on the SLB in the presence of ATP and remain stationary in AMPPNP. Since motor detachment from the microtubule is inhibited by AMPPNP, the steady-state accumulation signal at a given motor density is stronger in AMPPNP than in ATP.

QUANTIFICATION AND STATISTICAL ANALYSIS

Measure motor accumulation intensity

1. Open a video in Fiji and find a microtubule that lands after the video starts (Figure 5A).
2. Create a max projection of the video (Image -> Stacks -> Z project, Projection type: Max Intensity). This projection of the accumulated motors shows the trajectory of the microtubule (Figure 5B).
3. Synchronize the max projection image and the original video (Analyze -> Tools -> Synchronize Windows, select the files by clicking their names while holding the Shift key).
4. Use the *Segmented line* selection tool to make a selection along the accumulation trajectory on the max projection image (Figure 5C). Choose a line width just sufficient to cover the accumulation signal.

5. The line selection should be synchronized to the original video. Select the original video, and run the macro “LineScanOverTime” (See Data and Code Availability). This will generate a table of intensity values (Figure 6A), where each column contains the intensity values along the line selection in one frame.

6. Copy the data to an Excel spreadsheet, make sure to delete the first column that contains the pixel numbers (Figure 6B).

7. Repeat this analysis for other events under the same condition. Organize the data in one Excel workbook, where each sheet contains data for one accumulation event (Figure 6B).

**Fit accumulation rates**

8. Run “Accumulation_GUI” (see Data and Code Availability) in MATLAB (Figure 7).

9. Click **Import** and select the Excel workbook containing the data.

10. This MATLAB code provides two methods for intensity normalization to account for uneven illumination in the imaging field (as seen in the Raw intensity in Figure 7). The background intensity for each pixel is individually calculated, and the intensity value of each pixel is subtracted by its corresponding background value throughout the entire duration of the accumulation trace.
   a. The **Background median** option defines background as the median intensity before a microtubule lands. This is the default option that we choose for analyzing the accumulation data, and it works well for most cases (99% of the time), when there are sufficient data points before a microtubule lands so the median intensity can be calculated. However, this option does not work as well in the very rare cases when a microtubule lands very early in the video or if there are intensity fluctuations before a microtubule lands, therefore we add the **Lower 10%** option as an alternative.
   b. The **Lower 10%** option defines the background as the 10th percentile of the intensity in each pixel.

11. Fill out Time interval and Pixel size.

12. In **MT Landing – Rough Estimation** panel:
   a. The default frame range is the maximum range for the selected microtubule, adjust if needed. The beginning frame is relevant for background correction, since any data before the beginning frame is not considered, therefore if there are fluctuations in the background signal the trace can be truncated. The last frame is relevant for fitting the accumulation curve. If there are intensity fluctuations when the signal reaches the plateau, the trace can also be truncated.
b. Motor accumulation signal follows an exponential increase, which has a signature that the slope maximum is found at the beginning of the trace. We use this property to find the frame when a microtubule lands. In this step, the slope is calculated simply by dividing the difference of average raw intensity values in adjacent frame windows by the specified time window, thus this is a rough estimation. Define the frame window to find the maximum slope of the raw intensity versus time trace. A frame window between 10 to 20 works well under our experimental conditions.

c. Click **Intensity vs Time**, a graph should appear as shown in Figure 7. The gray curve shows the average raw intensity of all pixels over time, and the black trace shows the smoothed curve using the time window defined in the last step. These two curves are plotted on the primary y-axis on the left. The orange trace, plotted on the secondary y-axis on the right, shows the slope over time. A spike in the orange trace should overlap with the beginning of the intensity increase in the gray and black traces. Additionally, a kymograph generated from the raw data will appear under **Images Raw intensity for visualization**.

13. In **MT Landing – Fine Tune** panel, a local zoom in of the raw intensity vs time trace around where the maximum slope is found should now appear. Enter the frame window for fine tuning the calculation of the maximum slope by performing linear regression in each time window. Click **Find**, a graph and the frame in which a microtubule lands should appear as shown in Figure 7. The raw intensity trace is shown in blue and the slope over time is shown in orange. Once the microtubule landing frame is defined, a corrected kymograph should appear under **Images Normalized intensity**.

**Note:** The frame window used is largely dependent on the accumulation rate under specific conditions. For accumulation in ATP, which reaches steady state more quickly, a short frame window, such as 5–10, is ideal. In AMPPNP, accumulation happens more slowly, a longer frame window, such as 20, works better. When accumulation is carried out at low motor densities in AMPPNP, an even smaller frame window such as 30 may be needed.

14. In the **Accumulation Rate Fitting** panel, obtain the accumulation rate by defining the criteria for accepting a fit.
   a. We typically accept a fit when the total duration of the trace exceeds 3× time constant (1/rate constant) from the fit, and the $R^2$ of the fit must exceed 0.7.
   b. Once **Fit** is clicked, the code will use the frame in which a microtubule lands as time 0, and fit the normalized average intensity over time to a rising exponential. It starts fitting 50 frames in
total, then increases the fitting duration by 10 frames in each iteration until the criteria are met. Once the criteria are met, the final fit and the data used for fitting will be displayed and the fitting parameters will be saved in the program.

c. If desired, the displayed graph can be exported by clicking Save. This will save the figure into a subfolder called Graphs under the folder where the code is located.

d. If the criteria are not met after all possible fits are examined, a graph for the last fit will still be displayed but the fitting parameters will not be saved. Troubleshooting 4 and 5

e. If a fit is accepted by the program but a visual inspection indicates anomalous fluctuations in the intensity trace, the fitting information that the program has saved can be removed by clicking Abort.

f. If the data for generating the plot is needed, click Export Points. This will generate a MATLAB file Data4Fitting.mat that contains time, raw intensity data, normalized intensity values, and the fitting parameters.

15. Repeat steps 10–14 until all the data in the imported workbook are fit. Click Export in the General panel. This will export an Excel workbook Result.xls in a subfolder named Tables under the folder containing the code. The first sheet in the workbook contains the fitting information for all the fits that meet the criteria. A blank row indicates the fit does not meet the criteria. F_tot is the total frame number fitted. k_acc, a, c are parameters obtained by fitting the normalized average intensity data to \(-a/e^{k_{acc}t} + c\).
Calculate $k_{\text{on} \ 2D}$ and $k_{\text{off}}$ by performing a linear regression between accumulation rate and motor density

Motor accumulation is a reversible biomolecular association reaction (Pollard and De La Cruz, 2013). Therefore, $k_{\text{acc}} = k_{\text{on} \ 2D} \cdot [\text{Kin}]_{\text{tot}} + k_{\text{off}}$ (Jiang et al., 2019). When plotting accumulation rate ($k_{\text{acc}}$) against surface motor density ($[\text{Kin}]_{\text{tot}}$), the data should follow a linear relationship (Figure 8). When a linear regression is performed, the slope represents the association rate $k_{\text{on} \ 2D}$ and the y-intercept equals to the dissociation rate $k_{\text{off}}$.

LIMITATIONS

The key to successfully measuring motor binding kinetics by this 2D accumulation assay is that the motors generate measurable signal along the microtubule compared to the background signal from motors on the SLB. Thus, motors that have low microtubule affinity may not provide sufficient accumulation signal in saturating ATP. And at high motor densities, the accumulation signal may be small relative to the high background fluorescence. In our study (Jiang et al., 2019), kinesin-1 has the same $k_{\text{on} \ 2D}$ in ATP and AMPPNP (Figure 8). For motors with similar chemomechanical cycles in which motor association rate is not dependent on nucleotide condition, $k_{\text{on} \ 2D}$ can still be measured in AMPPNP. Motor dissociation rate can alternatively be measured by single-molecule motility assay (Feng et al., 2018).

The accumulation rate may become challenging to measure under certain conditions even if the signal-to-noise ratio is sufficient. To measure extremely fast accumulation rates, the videos need to be taken at a fast frame rate to generate enough data points to be fitted to an exponential function. Since the fluorescence intensity scales with exposure time, this also requires the camera to have a very high sensitivity. If the accumulation rate is extremely slow, photobleaching of the motors may result in an intensity increase that does not follow a single rising exponential and fitting the data to obtain the accumulation rate will be difficult.

TROUBLESHOOTING

Problem 1

SLB or motor signal is not homogeneous at the beginning of the experiment. Slow or no recovery is observed when performing fluorescence recovery after photobleaching (FRAP) on the SLB.

Potential solution

Since the rupture of SUVs and their fusion into a homogeneous SLB rely on having a clean hydrophilic and flat surface, any unwanted materials on the coverslip can cause SLB defects. Moreover, the amphipathic nature of phospholipids makes SLB susceptible to contaminations of any amphipathic or hydrophobic chemicals in the assay solutions. If SLB defects are frequently observed in experiments, the easiest solution is usually to purchase/remake all the reagents used in the system. The order of steps that we typically take to solve this problem is as follows:
Check the SLB quality right after it is formed (step 5). Let it remain in buffer and do not include any other assay components. If the SLB shows defects at this stage, there is a problem with either the coverslips or the SUVs. a) Clean a new batch of coverslips. The previous ones might not have been cleaned properly or have become contaminated during storage. Check the hydrophilicity of the coverslips after cleaning. A water droplet should spread on the surface extremely easily and has a contact angle close to 0 (flat on the surface). Clean the coverslip by a plasma cleaner right before forming the SLB to remove any contaminants collected during storage. b) Make new MLVs and extrude new SUVs. c) Make new buffer.

If SLB quality is good in the above step, the problem likely involves one or more reagents in the assay solutions. Since it is unclear whether the problem is caused by one component or the interaction between two or more components, it is usually easier to switch all the reagents instead of testing them individually/combinatorially. In our experience, defects in lipid bilayer are commonly caused by the presence of amphipathic or hydrophobic chemicals in the assay system.

Detergents disrupt lipid bilayer structure and should be avoided in the assay system. Double-sided tape should not be used as a spacer for the assay chamber since the adhesive on the tape can dissolve and enter the assay chamber after repeated washing.

Caution needs to be taken when using drugs with poor water solubility in this assay. In our case, the microtubule-stabilizing drug, taxol, is extremely insoluble in water. Therefore, the stock solution is made in DMSO. Both taxol and DMSO can change the properties of the lipid membrane; thus, it is important to use the lowest possible concentration of taxol on SLBs. Microtubules are stabilized by 10 μM taxol upon polymerization, but the motility buffer for the accumulation assay on SLBs only contains 1 μM taxol. Since microtubules are added immediately before imaging and only imaged for a very short amount of time, we have not seen any problems of microtubule depolymerization during the course of the assay.

Denatured proteins are another source of hydrophobic reagents in this assay. Improper storage of proteins, such as repeated freeze-thaw cycles, can lead to protein denaturation. Denaturation leads to the exposure of hydrophobic residues that are normally buried when the protein is properly folded. The denatured protein may directly cause membrane defects, or indirectly through the accumulation of undesired by-products arising from the disrupted enzymatic pathways. For example, in the glucose/glucose oxidase/catalase oxygen scavenging system, denaturation of catalase will cause a buildup of hydrogen peroxide, which is detrimental to both the SLB and the kinesin motors.

Problem 2
Unable to detect motor accumulation.

Potential solution
Since the signal-to-noise ratio is key to the success of the accumulation assay, we always purify active motors by microtubule pelleting assay right before running the experiments. Otherwise, the inactive motors can substantially increase the background, which not only makes accumulation difficult to visualize but also causes inaccurate estimation of motor density. If both the SLB and the kinesin motors on the surface show homogeneous fluorescence but weak or no accumulation is observed, the motors may have lost activity. In this case, motor activity can be checked by an ATPase assay before running the accumulation assay. If motors have normal activity after the microtubule pelleting assay but still give weak accumulation signal on the SLB, go through the checklist in Troubleshooting 1 to see if any components of the assay solutions have gone bad.

Problem 3
GFP intensity and motor density do not follow a linear relationship.
Potential solution
Potential causes of this problem: 1) an improper exposure time is used; 2) The amount of kinesin-NeutrAvidin incubated with the SLB is not sufficient to saturate all the binding sites.

When imaging motor fluorescence, if the exposure time is too short, the intensity values at extremely low motor densities may not differ; if the exposure time is too long, the signal may saturate at high motor densities. To find a proper exposure time, image motor signal at the two lowest motor densities, and choose the minimum exposure time that provides different intensity values for the two motor densities. If this minimum exposure time still causes the signal to saturate at high motor densities, reduce the laser power and repeat the above step again.

If the yield of kinesin-NeutrAvidin complexes from the microtubule pelleting assay is low, the incubation step can be repeated one more time with new solution. Or the concentration of motor, NeutrAvidin and microtubules used in the pelleting assay can be increased to 400 nM, 4 μM, and 1.5 μM, respectively. If the above two steps do not provide full motor coverage on the SLB, the motor prep may have extremely low activity or is poorly biotinylated. In this case, purify a new motor prep. For kinesin constructs with a C-terminal AviTag, we supplement 0.6 mM biotin to the medium at induction.

Problem 4
Motor accumulation signal does not follow a single exponential rise.

Potential solution
If a stable plateau is not observed, it is likely due to photobleaching of GFP. In this case, perform the accumulation assay with reduced laser power. Additionally, the accumulation trace may deviate from an exponential function if the underlying assumption that free motor density on the membrane remains constant is not met, in which case the microtubule concentration needs to be reduced or the motor density needs to be increased.

Problem 5
There is no accepted fit that satisfies the defined criteria.

Potential solution
If issues stated in Problem 4 are excluded but there is no acceptable fit, it is likely that the imaging parameters need to be adjusted. The $R^2$ will exceed the cutoff if there are not enough data points to constrain the fit, in which case a faster frame rate is needed. The program can also reject the fit if the trace is too short and does not last longer than 3 $\times$ time constants, in which case the total video length needs to be increased.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, William O. Hancock (woh1@psu.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The Fiji macro and MATLAB codes for data analysis, as well as an example data spreadsheet with the output are available to readers in the Pennsylvania State University institutional repository, ScholarSphere, https://doi.org/10.26207/hetk-tc10.
SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100691.

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AUTHOR CONTRIBUTIONS
Conceptualization, R.J. and W.O.H.; methodology, R.J.; software, R.J.; investigation, R.J.; writing – original draft, R.J.; writing – review & editing, W.O.H.; funding acquisition, W.O.H.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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