Chapter 14

Measurements of the Force-Dependent Detachment Rates of Cytoplasmic Dynein from Microtubules

Xinglei Liu, Lu Rao, and Arne Gennerich

Abstract

Cytoplasmic dynein, the largest and most intricate cytoskeletal motor protein, powers the movement of numerous intracellular cargos toward the minus ends of microtubules (MT). Despite its essential roles in eukaryotic cells, dynein's molecular mechanism, the regulatory functions of its subunits and accessory proteins, and the consequences of human disease mutations on dynein force generation remain largely unclear. Recent work combining mutagenesis, single-molecule fluorescence, and optical tweezers-based force measurement have provided valuable insights into how dynein's multiple AAA+ ATPase domains regulate dynein's attachment to MTs. Here, we describe detailed protocols for the measurements of the force-dependent dynein-MT detachment rates. We provide updated and optimized protocols for the expression and purification of a tail-truncated single-headed *Saccharomyces cerevisiae* dynein, for polarity-marked MT polymerization, and for the non-covalent attachment of MTs to cover glass surfaces for the measurement of dynein-MT detachment forces.

Key words Microtubules, Microtubule motor proteins, Cytoplasmic dynein, Recombinant proteins, Microtubule immobilization, Fluorescence labeling, Single-molecule assays, Optical tweezers, Optical trapping, Yeast gene manipulation

1 Introduction

Cytoplasmic dynein (referred to here as dynein) uses the energy of ATP hydrolysis to drive nearly all microtubule (MT) minus-end-directed motility in eukaryotes [1–4]. It is composed of two identical heavy chains (HCs) and several subunits and accessory proteins that regulate dynein function and cargo binding [4]. The dynein complex is responsible for a variety of cellular functions, including the transport of organelles and mRNAs, the transport of nuclei during neuronal migration, and the formation and positioning of the mitotic spindle (reviewed in refs. 1–4). The dynein HC contains a dimerizing N-terminal tail domain and a C-terminal motor

Steven M. Markus (ed.), *Dynein: Methods and Protocols*,
Methods in Molecular Biology, vol. 2623, https://doi.org/10.1007/978-1-0716-2958-1_14,

domain (MD) or "head" with six tandem-linked AAA+ ATPase modules (AAA: ATPase associated with various cellular activities) arranged in a ring (AAA1-6). AAA1, 3, and 4 are the only active ATPases [1]. Three elongated structures emerge from the AAA ring: a ~ 15-nm coiled-coil "stalk" that protrudes from AAA4 and separates dynein's MT-binding domain (MTBD) from the AAA+ ring [5, 6]; the buttress [5] (or strut [6]), which is an antiparallel coiled-coil that emerges from AAA5 and contacts the stalk; and a ~10-nm "linker" that extends from AAA1 and connects the AAA+ ring to the tail [7–9]. The linker undergoes conformational changes [7, 10, 11] that are essential for the generation of unidirectional motion and force [12], and it also controls the buttress-mediated sliding of the stalk helices to shift between weak and strong MT-binding states [13].

In contrast to mammalian dynein, which needs to associate with its largest subunit dynactin and a coiled-coil cargo adaptor protein to move processively along MTs [14, 15], *Saccharomyces cerevisiae* dynein is capable of moving over micrometer distances on its own [16], with one head taking a forward step while its identical partner head stays MT bound. Combining mutagenesis with single-molecule fluorescence and optical tweezers-based force measurements, recent work has provided insights into how dynein keeps its two MDs "out-of-phase" to ensure that one MD stays MT bound while the other MD detaches and advances [13, 17–20]. The three active ATPases (AAA1, 3, and 4) work together to control the cyclic MT attachment and detachment cycles of dynein's leading and trailing MDs under directional load [13, 17, 18]. While the primary ATPase AAA1 is essential for dynein activity [21], AAA3 and AAA4 control the effects of AAA1-ATP binding and hydrolysis on both dynein-MT binding and on linker conformational changes [13, 17, 18, 20].

In this chapter, we provide improved protocols for the measurement of the MT detachment rates of a *S. cerevisiae* dynein MD. In addition, we present updated protocols for the expression and purification of single-headed *S. cerevisiae* dynein and for the preparation of polarity-marked MTs. We further describe an improved protocol for the non-covalent immobilization of MTs on cover glass surfaces. The presented protocol is easy to follow and prevents interactions between trapping beads and the cover glass surface, which would otherwise result in the measurement of non-specific unbinding forces. Besides the measurement of the direction-dependent MT-binding strength of dynein, the provided protocols are applicable to a wide range of MT-associated motors and MT-associated proteins (MAPs).

2 Materials

2.1 Yeast Growth and Protein Expression

1. Yeast stock, stored at −80 °C.
2. Puritan™ wood applicator stick (Fisher Scientific #22029680).
3. 50 mL Falcon conical centrifuge tube.
4. Peptone: Bacto™ peptone, an enzymatic digest of animal protein (ThermoFisher Scientific, #211677).
5. Yeast extract: Bacto™ yeast extract, water-soluble portion of autolyzed *Saccharomyces cerevisiae* cells suitable for use in culture media (ThermoFisher Scientific, #212730).
6. 2× YP solution: Add 80 g peptone, 40 g yeast extract, and 1.75 L ddH_2O into a 2-L flask, autoclave and store at room temperature.
7. Dextrose (D-glucose), anhydrous.
8. Dextrose solution: 40% stock. Add 400 g of dextrose to a 1-L bottle, filled with ddH_2O to 1 L, sterilize by filtering via a filter unit with 0.2 μm pore size.
9. 2× YPD solution: Add 80 g peptone, 40 g yeast extract, and 1.75 L ddH_2O in a 2-L flask, then autoclave. Once slightly cooled down, add 200 mL of sterilized dextrose solution. Mix well.
10. Agar.
11. Petri dish: Sterilin™ Standard 90 mm Petri dish (ThermoFisher Scientific, #101IRR).
12. YPD plate: For 100 mL, add 2 g peptone, 1 g yeast extract, 2 g agar and 85 mL ddH_2O to a 150-mL bottle, autoclave. Once slightly cooled, add 5 mL of sterilized dextrose solution, mix well. Pour 10 mL for each plate. Stack plates and leave on the bench top overnight. Store upside down in sterilized bag at 4 °C.
13. D-(+)-Raffinose pentahydrate.
14. Raffinose solution: 20% stock. Add 20 g raffinose to a 150 mL bottle, filled with ddH_2O to 100 mL. Slightly heat to dissolve. Sterilize via filtering through a 0.2 μm filter.
15. 1× YPR solution: Add 20 g peptone, 10 g yeast extract, and 900 mL ddH_2O in 1 L bottle, autoclave. Once slightly cooler, add 100 mL of sterilized raffinose solution. Mix well.
16. D-(+)-Galactcose.
17. Galactose solution: 40% stock. Add 400 g galactose to a 1-L bottle, filled with ddH_2O to 1 L, heat to dissolve. Sterilize via filtering through a 0.2 μm filter.
18. 1 L centrifuge bottle: Nalgene™ 1 L super-speed centrifuge bottles with sealing closure (ThermoFisher Scientific, #3140-1006).

2.2 Yeast Dynein Purification

1. HEPES (N-2-hydroxyethylpiperazine-N-2-ehtane sulfonic acid): a zwitterionic organic chemical buffering agent commonly used in cell culture media.
2. DTT (dithiothreitol): quantitatively reduces disulfide bonds and maintains monothiols in a reduced state.
3. ATP.Mg (Adenosine 5′-triphosphate magnesium salt): Dissolve ATP.Mg in ddH_2O to 100 mM stock (Sigma-Aldrich, #A9187).
4. Pepstatin A: reversibly inhibits aspartic acid proteases. Dissolve in ddH_2O to 10 μg/mL stock, store at −20 °C (ThermoFisher Scientific, #78436).
5. Leupeptin: reversibly inhibits serine and cysteine proteases. Dissolve in ddH_2O to 10 μg/mL stock, store at −20 °C (ThermoFisher Scientific, #78435).
6. Pefabloc (AEBSF): irreversible inhibitor of serine proteases. Dissolve in ddH_2O to 100 mM stock, store at −20 °C (ThermoFisher Scientific, #78431).
7. Benzamidine hydrochloride, hydrate: dissolve in ddH_2O to 200 mM stock, store at −20 °C (Sigma-Aldrich, #12072).
8. Triton X-100: dilute to 25% (v/v) stock (Sigma-Aldrich, #9002-93-1).
9. 5× Lysis Buffer (5× LB buffer): 150 mM HEPES, 250 mM KAc, 10 mM $Mg(AC)_2$, 5 mM EGTA, 50% Glycerol.
10. Coffee grinder.
11. Beckman Optima TLX Ultracentrifuge (Beckman Coulter).
12. Beckman TLA-110 rotor: fixed angle (Beckman Coulter, #366735).
13. TLA-110 tube: Polycarbonate tube, 3.2 mL, 13 × 56 mm (Beckman Coulter, #362305).
14. Econo-Pac Chromatography column: used for gravity-flow chromatography, 1.5 × 12 cm, polypropylene columns (BIO-RAD, #7321010).
15. Serological pipets: disposable polystyrene pipets, sterile, 50 mL.

2.2.1 Dynein Purification via ZZ-Tag

1. IgG Sepharose 6 Fast Flow: allows high-flow rates (Millipore Sigma, #GE17-0969-01).
2. HaloTag fluorescent ligands (Promega) or SNAP-tag fluorescent ligands (New England BioLabs).
3. TEV buffer (TEV): 50 mM HEPES, 150 mM KAc, 2 mM $Mg(Ac)_2$, 1 mM EGTA, 10% Glycerol.
4. TEV protease: AcTEV™ protease, removes affinity tags from fusion proteins, stored at −20 °C (ThermoFisher Scientific, #12575023).

2.2.2 MT-Binding and -Release Purification

1. Paclitaxel (taxol): 10 mM in DMSO, stored at −20 °C (Sigma Aldrich, #T7402).
2. 25% Sucrose cushion: 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 25% sucrose, 10% glycerol, pH 7.4.
3. Wash buffer 1 (WB-1): 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, 150 mM KCl, 10% glycerol, pH 7.4.
4. Unlabeled MTs, 5 mg/mL.
5. TLA-100 fixed angle rotor (Beckman Coulter, #343840).
6. TLA-100 rotor tube: polycarbonate tube, 7 × 20 mm (Beckman Coulter, #343775).
7. SDS-PAGE gel: 4–12%, Bis-Tris.
8. 5× SDS loading buffer: 0.25% (w/v) bromophenol blue, 10% (w/v) sodium dodecyl sulfate (SDS), 0.5 mM DTT, 0.25 M Tris, 50% (v/v) glycerol, pH 6.8.
9. Acrylamide gel electrophoresis system.
10. SDS-PAGE running buffer.
11. InstantBlue Coomassie Protein Stain, or similar.
12. Gel imaging system.

2.3 Flow Chamber Preparation

2.3.1 Coverslip Cleaning

1. Coverslips: precision cover glasses of a thickness of 170 ± 5 μm, 18 × 18 mm, high performance (Marienfeld, #0107032).
2. Coverslip rack (Thomas Scientific, #8542E40-PKOF6).
3. Superfrost™ plus microscope slides: 25 × 75 mm (Fisher Scientific, #12-550-15).
4. 25% HNO_3.
5. 2 M NaOH.

2.3.2 Polymerization of Polarity-Marked MTs

1. GTP solution (100 mM) (ThermoFisher Scientific, #R0461).
2. BRB80 with 10% glycerol: 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, pH 6.8.
3. BRB80 with 60% glycerol: 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EDTA, 60% glycerol, pH 6.8.
4. Tubulin (Rhodamine labeled): Porcine brain tubulin modified to contain covalently linked rhodamine at random surface lysines (Cytoskeleton, #TL590M).
5. Tubulin (Biotin tagged): Porcine brain tubulin modified to contain covalently linked, long-chain biotin derivative (Cytoskeleton, #T333P).
6. Tubulin (>99% Pure): white lyophilized powder, 1 mg. Dissolve in 100 μL BRB80 + 10% glycerol and aliquot to 20 × 5 μL (final 10 mg/mL) (Cytoskeleton, #T240).

2.3.3 MT Immobilization

1. HME30 buffer: 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2.
2. HME30 with 10% glycerol buffer: 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, pH 7.2.
3. α-casein: dissolve lyophilized powder (Sigma Aldrich, #C6780) in HME30 with 10% glycerol to a final concentration of 25 mg/mL.
4. EZ-Link™ Sulfo-NHS-Biotin: a short-chain, water soluble biotinylation reagent for labeling antibodies, proteins and other molecules that have primary amines, 1 mg. (ThermoFisher Scientific, #A39256).
5. Amicon™ Ultra Centrifugal Filter Units: regenerated cellulose membranes that are ideal for protein and nucleic acid purification, concentration, and desalting. 10 kDa, 0.5 mL (Millipore Sigma, #UFC501024).
6. Humidity box: place damp tissue in a box with tight cover.
7. Pluronic F-127: dissolve powder in ddH_2O to a final concentration of 10% (w/v).
8. BRB80 with 1% Pluronic F-127: 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA, 1% Pluronic F-127, pH 6.8.
9. Wash buffer 2 (WB-2): add 2 μL of 25 mg/mL biotinylated α-casein (0.25 mg/mL final) and 8 μL of 25 mg/mL α-casein (1 mg/mL final) to 190 μL of BRB80 with 1% Pluronic F-127, pH 6.8.
10. Blocking buffer (BB): add 6 μL of 25 mg/mL α-casein (0.75 mg/mL final) to 194 μL of BRB80 with 1% Pluronic F-127 and add taxol to 10 μM final.
11. Streptavidin: dissolve powder in BRB80 with 10% glycerol to a final concentration of 1 mg/mL (ThermoFisher Scientific, #21122).
12. 0.2 mg/mL biotinylated MTs: prepared as described previously in detail [22].

2.4 Unbinding-Force Assay

1. TCEP: dissolve Tris(2-carboxyethyl)phosphine hydrochloride (a strong reducing agent) in ddH_2O to a final concentration of 100 mM, and store at −20 °C.
2. Motility buffer (MB): Dissolve glucose powder in 50 mL HME30 with 0.5% Pluronic F-127 to a final concentration of 20 mM, and store at 4 °C. Prepare 200 μL MB by pipetting 6 μL of 25 mg/mL α-casein (0.75 mg/mL final) and 1 μL of TCEP (1 mM final) to 193 μL of HME30 with 0.5% Pluronic F-127 containing 20 mM glucose solution, keep it on ice. Use within 1 h.

3. Biotin: dissolve powder in ddH_2O to a final concentration of 50 mM, and store at −20 °C.
4. Glucose oxidase: dissolve lyophilized powder in 50 mM HEPES with 50% glycerol to a final concentration of 10 μM (Millipore Sigma, #C40), and store at −20 °C. Use within 1 month.
5. Catalase: dissolve lyophilized powder (from bovine liver) in 50 mM HEPES with 50% glycerol to a final concentration of 5 μM (Millipore Sigma, #G2133). Store at −20 °C and use within 1 month.
6. Gloxy: prepared by mixing glucose oxidase and catalase in 1:1 ratio (v/v). Store at −20 °C and use within a week.
7. Apyrase: active ATP-diphosphohydrolase that catalyzes the hydrolysis of ATP to AMP releasing inorganic phosphate, 500 units/mL (New England BioLabs, #M0398S).
8. C-Trap™ Optical Tweezer (Lumicks).

3 Methods

3.1 Yeast Protein Expression and Cell Harvest

This protocol describes the expression of yeast dynein induced by galactose via the GAL1 promoter.

1. Take out a tube with the yeast strain glycerol stock from the −80 °C freezer and place it on dry ice to prevent melting. Use a sterile wood applicator stick to scrape yeast cells from the tube and streak onto a YPD plate. Incubate the parafilm sealed plate at 30 °C for 48–72 h until colonies are large enough (~1–2 mm in diameter) (*see* **Note 1**).
2. Use a sterile pipette tip to pick a single colony from the plate and drop it into a 50-mL Falcon tube containing 3–5 mL of 2× YPD solution to start the inoculation. Allow cells to grow overnight at 30 °C with shaking.
3. Transfer the overnight culture into 45 mL of 1× YPR solution. Incubate for 8 h at 30 °C with shaking.
4. Add the 50 mL culture into 1.75 L of 2× YP solution and add 200 mL sterilized galactose solution into the flask. Incubate for 18–24 h at 30 °C with shaking.
5. Transfer cell culture to two 1 L centrifuge bottle. Harvest cell culture by centrifugation at 500× *g* for 5 min at 4 °C (*see* **Note 2**).
6. Discard the supernatant and resuspend the cell pellet with 500 mL ddH_2O. Centrifuge again at 500× *g* for 5 min at 4 °C to harvest cells.

7. Carefully discard the supernatant. Resuspend the cells with a 50 mL serological pipette using the residual water. If necessary, add limited amount of ddH_2O until the pellet can be resuspended (*see* **Note 3**).
8. Drip the cell slurry dropwise into liquid nitrogen using the 50 mL serological pipette (*see* **Note 4**). Transfer the popcorn-like frozen droplets into a 50 mL Falcon tube using a chilled metal spoon. Store the tube at −80 °C until you are ready for the next step. Note that the frozen cells are stable for a few months in the −80 °C freezer.

3.2 Yeast Dynein Purification

The purification of the single-headed yeast dynein (Dyn_{331kDa}) is done in two steps: first, the protein is purified via a ZZ-tag at the N-terminus, which is followed by a TEV cleavage step [16]; second, sequential MT-binding and -release steps are performed [22]. In the first step, the ZZ-tagged dynein is isolated from cell lysate by the binding to IgG beads, which is subsequently released from the IgG beads with TEV protease. The second step helps remove non-functional and/or aggregated dynein motors and other impurities through MT-mediated pulldown and ATP-induced release.

3.2.1 Dynein Purification via ZZ-Tag

This is the first step in the purification of dynein. **Steps 1–14** describe how to prepare and clarify the cell lysate, while **steps 15–29** describe how to purify dynein via the ZZ-tag (*see* **Note 5**).

1. Chill all buffers on ice. Add DTT (1 mM final), ATP.Mg (100 μM final), pepstatin A (10 ng/mL final), leupeptin (10 ng/mL final), pefabloc SC (0.5 mM final), benzamidine (8 mM final), and 1.92 mL ddH_2O to 12 mL 5× LB buffer to yield 15 mL of 4× LB buffer.
2. Add 6 mL of 4× LB buffer to 18 mL ddH_2O to obtain 24 mL of 1× LB.
3. Add (v/v) 0.2% final Triton X-100 to the remaining 9 mL of 4× LB buffer (*see* **Note 6**).
4. Prechill a TLA-110 rotor, 8–12 TLA-110 rotor tubes, a 100 mL glass beaker, a 50-mL serological pipette, two gravity columns, and a 50 mL Falcon tube in the refrigerator. Set the Beckman Optima TLX ultracentrifuge to 4 °C.
5. Chill the coffee grinder and its plastic lid with liquid nitrogen.
6. Take out one tube of frozen cell droplets from the −80 °C freezer (corresponding to 2 L of cell growth) and transfer the droplets into the pre-chilled grinder. Start grinding to break the cell droplets and stop grinding as soon as a sign of melting appears on lid (*see* **Note 7**).
7. Transfer the ground powder with a chilled metal spatula into the prechilled 100 mL glass beaker and add immediately 3 mL

of 4× LB buffer to the powder, then transfer the beaker to a 37 °C bath.

8. Gently stir the powder with a plastic pipette to thaw it evenly. Transfer the beaker quickly back on ice when only a small amount of ice left (close to completely thawed).
9. Estimate the volume of the cell lysate by using the prechilled 50 mL serological pipette, then add a sufficient volume of 4× LB solution to achieve a final 1× LB concentration.
10. Transfer thawed cell lysate to 8–12 rotor tubes (*see* **Note 8**). Keep tubes on ice.
11. Place tubes into the TLA-110 rotor and place the rotor in the Beckman Optima TLX ultracentrifuge.
12. Centrifuge at 80,000 rpm for 10 min at 4 °C.
13. Once the centrifugation has finished, put tubes quickly back on ice. Transfer the supernatant into the prechilled 50-mL Falcon tube (*see* **Note 9**).
14. Estimate the volume of the lysate and add ATP.Mg to a final concentration of 0.1 mM and pefabloc SC to a final concentration of 0.5 mM.
15. Take the IgG beads from the refrigerator. Transfer 250 μL bead slurry into the prechilled chromatography column (*see* **Note** 10). Wash twice with 2.5 mL of 1× LB buffer by letting the liquid drain through the membrane at the bottom of the column. Cap the bottom of the column when the solution containing bead slurry in the column decreases to ~500 μL.
16. Transfer the ~500 μL bead solution into the 50-mL Falcon tube containing the cell lysate.
17. Seal the tube with parafilm and nutate it for 1 h at 4 °C.
18. Following nutation, transfer the solution into a prechilled gravity column. Remove the bottom cap to allow the solution to run through the column.
19. Wait until the solution level is close to the bead surface. Wash the beads with 5 mL 1× LB buffer. Repeat this step twice.
20. When a ~350 μL solution remains (~100 μL remains on top of the beads), cap the end of the column.
21. Label the protein by adding HaloTag Ligand or SNAP-tag Ligand to the column (~10 μM final) (*see* **Note 11**).
22. Incubate at room temperature for 10 min or at 4 °C for 2 h. Gently stir the beads during incubation using a chilled metal spatula.
23. After the incubation, remove the cap from the bottom. Once the solution has drained completely, wash the beads with 10 mL 1× LB buffer.

24. Wash the beads again with 3 mL 1× TEV buffer. Repeat this step twice. Cap the bottom of the column when ~200 μL solution remains.
25. Transfer the solution into a 2 mL low-retention microcentrifuge tube. Add limited amount of 1× TEV buffer to the column, resuspend the remaining beads, and transfer them to the tube.
26. Let the beads settle for 5 min, then remove the supernatant until ~400 μL solution is left. Add 4 μL AcTEV™ protease to the tube.
27. Nutate the tube at 4 °C for 2 h.
28. Centrifuge the tube at 1000× *g* for 30 s at 4 °C. Transfer the supernatant to a 0.5 mL low-retention microcentrifuge tube. Repeat the step to get the entire supernatant (*see* **Note 12**).
29. Aliquot into 50 μL volumes (solution with the TEV-released dynein). Flash-freeze the protein using liquid nitrogen. Store at −80 °C.

3.2.2 MT-Binding and -Release Purification

1. Add DTT (1 mM final) and taxol (20 μM final) to 100 μL 25% sucrose buffer and mix well.
2. Add DTT (1 mM final) and taxol (20 μM final) to 200 μL WB-1 and mix well.
3. Thaw an aliquot of 50 μL solution with the TEV-released dynein solution on ice. Transfer 5 μL of the dynein solution into a new tube and label the tube with an "I" (Initial Sample) for a gel.
4. Supply the dynein solution with taxol (20 μM final) and mix well. Warm it to room temperature.
5. Add 5 μL of unlabeled MTs to the dynein solution and mix well by gentle pipetting. Incubate at room temperature for 5 min to allow dynein to bind to MTs (*see* **Note 13**).
6. Add 100 μL of sucrose cushion to a TLA 100 centrifuge tube, mark the outside edge of the tube, and place the tube inside the TLA-100 rotor with the mark facing outward. Place a balance tube in the opposite rotor position.
7. Carefully pipet MT/dynein solution from **step 5** on the top of the sucrose cushion.
8. Centrifuge at 40,000 rpm (69,522× *g*) for 10 min at 25 °C.
9. Transfer 5 μL of the supernatant into a new tube and label the tube with "S1" (Supernatant 1). Remove the supernatant and cushion carefully.
10. Wash the pellet with 20 μL WB-1 and carefully remove the buffer.

11. Resuspend the pellet in 52 μL WB-1. Transfer 5 μL of the solution into a new tube and label the tube as "P1" (Pellet 1).
12. Add 6 mM final ATP.Mg to the resuspended pellet and carefully mix by inverting the tube. Incubate at room temperature for 2 min.
13. Centrifuge the sample at 40,000 rpm (69,522× *g*) for 5 min at 25 °C.
14. Transfer 5 μL of the supernatant to a new tube and label the tube as "S2" (Supernatant 2).
15. Aliquot the rest of supernatant into 1.5 μL volumes using PCR tubes. Flash-freeze the aliquots and store at −80 °C.
16. Wash the pellet carefully with 20 μL WB-1 and remove the WB-1.
17. Resuspend the pellet in 50 μL WB-1. Transfer 5 μL of the solution into a new tube and label the tube as "P2" (Pellet 2).
18. Mix the saved 5 μL samples ("I", "S1", "P1", "S2," and "P2") each with 2 μL 5× SDS loading buffer and add ddH_2O to a final volume of 10 μL. Boil each sample for 3 min. Load each sample onto a 4–12% acrylamide gel and fill the chamber with 1× running buffer. Run the gel for 40 min at 200 V. Stain the gel with InstantBlue Coomassie protein stain solution and scan the gel with an Odyssey imaging system (Fig. 1).

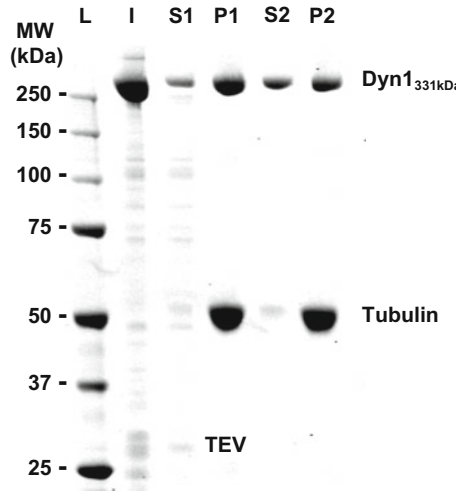

Fig. 1 ATPase activity-based purification of $Dyn1_{331kDa}$-GFP using a MT-binding and -release assay. 4–12% Bis-Tris SDS-PAGE stained with InstantBlue™. Input, 5 μL of a TEV-released dynein sample (initial sample); S1, 5 μL supernatant following spin; P1, 5 μL of re-suspended MT-pellet; S2, 5 μL of supernatant following a second spin; P2, 5 μL of MT-pellet re-suspended in 6 mM ATP containing buffer

3.3 Flow Chamber Preparation

This easy-to-follow protocol describes how to clean glass cover slips for the optical tweezers-based unbinding-force measurements using acid and base baths. Following the assembly of the flow chamber [23], functionalized (biotinylated) MTs are attached to the glass surface through a streptavidin-biotin linkage.

3.3.1 Coverslip Cleaning

1. Place coverslips in a coverslip rack and submerge the rack in 25% HNO_3 for 15 min.
2. Rinse coverslips with ddH_2O.
3. Submerge the rack in 2 M NaOH for 5 min.
4. Rinse extensively with ddH_2O.
5. Dry the coverslips on a heat block for 30 min.
6. Store the coverslips in a coverslip box and store at 4 °C. Use within 2 weeks.

3.3.2 Polymerization of Polarity-Marked MTs

1. Add 1 μL of 100 mM GTP to 99 μL BRB80 with 10% glycerol (BRB80G10) and keep the solution on ice.
2. Add 10 μL of BRB80G10 to one vial of Rhodamine tubulin to dissolve the tubulin and keep it on ice.
3. Add 20 μL BRB80G10 to one vial of biotin tubulin to dissolve the tubulin and keep it on ice for 5 min.
4. Add 5 μL of the dissolved biotin tubulin to 10 μL of dissolved Rhodamine tubulin and mix well. Keep it on ice.
5. Transfer 0.5 μL of the tubulin mixture each into 10 aliquots and label the aliquots with "S" for "Seed".
6. Mix 5 μL of the unlabeled tubulin, 5 μL of biotin tubulin, and the remaining ~10 μL tubulin mixture from **steps 4** and **5** and keep it on ice for 5 min.
7. Add 35 μL of BRB80G10 to the mixture and transfer into ten 5-μL aliquots. Label the aliquots as "P" for polymerization (*see* **Note 14**).
8. Incubate one aliquot of "S" at 37 °C for 5 min (*see* **Note 15**).
9. Warm one aliquot of "P" at 37 °C for 1 min and then add it to "S" from **step 8**. Gently mix the solution and incubate at 37 °C for 20 min (*see* **Note 16**).
10. Add 1 μL of 0.2 mM taxol (in DMSO) to 60 μL of BRB80 with 60% glycerol (cushion) and transfer the solution to a TLA100.1 tube.
11. Add 0.2 μL of 10 mM taxol (in DMSO) and 1 μL of 0.1 M DTT to 100 μL of BRB80 with 10% glycerol (hereafter referred to as BRB buffer).

12. Carefully layer the tubulin mixture on the top of the glycerol cushion in the TLA100.1 tube and spin at 80,000 rpm (278,088× *g*) for 10 min.
13. After spinning, remove the supernatant. Carefully wash the pellet twice by pipetting 20 μL BRB buffer onto the pellet and carefully remove it immediately afterwards.
14. Resuspend the pellet with 5 μL of BRB buffer by gentle pipetting.
15. Store the MT solution at room temperature in the dark (*see* **Note 17**).

3.3.3 MT Immobilization

1. Preparation of biotinylated α-casein: Add 200 μL of α-casein to one vial of sulfo-NHS-biotin and nutate at room temperature for 1 h.
2. Transfer the solution to a 0.5 mL 10 kDa Amicon™ ultra centrifugal filter. Add 300 μL of HME30 with 10% glycerol to the solution and spin at 14,000 *g* for 5 min. Repeat this step two more times. After last spin, spin the remaining solution and add a small amount of HME30 with 10% glycerol if necessary to obtain a 200-μL solution of biotinylated α-casein. Aliquot into 2 μL/vial and store at −20 °C.
3. Assemble a flow chamber as described in detail previously [23] using the cleaned coverslip prepared in Subheading 3.3.1.
4. Dilute 2 μL of biotinylated α-casein with 10 μL HME30 buffer and mix well. Flow the mixture into the chamber and incubate at room temperature for 10 min in a humidity box.
5. Wash the chamber three times with 20 μL WB-2, then incubate the flow chamber in a humidity box at room temperature for 30 min (*see* **Note 18**).
6. Completely remove the solution from the flow chamber using vacuum. Flow in 12 μL 1 mg/mL streptavidin solution and incubate at room temperature for 10 min in the humidity box.
7. Add 0.5 μL of polarity-marked MTs (final ~0.2 mg/mL) to 19.5 μL BB and mix well (*see* **Note 19**).
8. Completely remove the solution from the flow chamber. Wash twice with 20 μL BB.
9. Flow in the MT solution, then immediately wash with 2 × 20 μL BB (Fig. 2) (*see* **Note 20**).

3.4 Unbinding-Force Assay

The protocol below describes how to perform unbinding-force measurements on single-headed *S. cerevisiae* dynein using optical tweezers, which starts with coupling the N-terminal GFP-tag (immediately upstream of dynein's linker element) to an anti-GFP antibody-coated 0.5 μm polystyrene bead [24] (Fig. 3a, left).

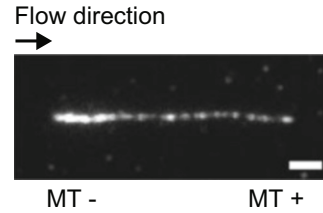

Fig. 2 TMR-labeled and polarity-marked MT bound to a cover glass via biotin-streptavidin linkages. The MT is aligned with the flow direction (image size: 178 × 89 pixels; pixel size: 110 nm). The bright MT minus end is on the left and the depicted horizontal scale bar corresponds to 2 μm

The beads are then introduced into the microscope chamber with the immobilized polarity-marked MTs (Fig. 2). The unbinding assay is performed with a C-Trap from LUMICKS but can also be performed with any other appropriately equipped and accurate optical tweezers setup [13, 17, 18]. Dynein-coated beads are captured with a fixed position optical trap and placed above a surface-bound MT (visualized via interference reflection microscopy or via fluorescence imaging) using the nano-positioning stage of the microscope. Upon MT binding, a monotonically increasing load is imposed on the bond between the MT and the dynein MTBD as a result of the continuously moving stage (depending on the direction of stage movement, the MT-MTBD bond experiences a MT plus- or minus-end-directed force) (Fig. 3a, right). Using this approach, the unbinding forces can be measured (Fig. 3b, left), and the force-dependent unbinding rates can be calculated [13, 17, 18] (Fig. 3b, right).

1. Dilute 0.5 μL of anti-GFP antibody-beads with 18.5 μL MB (*see* **Note 21**).
2. Thaw an aliquot of the dynein stock and pre-dilute the stock in MB as much as needed (typically by a factor of 10,000–50,000).
3. Mix 1 μL of pre-diluted motor with 19 μL MB containing trapping beads and incubate on ice for 10 min.
4. In the meantime, add 0.8 μL biotin and 0.8 μL gloxy to 20 μL MB. Add apyrase to the buffer to a final concentration of 6.6 units/mL (*see* **Note 22**).
5. Add the final 20 μL of motility buffer to 20 μL of the beads with the coupled dynein molecules and gently mix by pipetting.
6. Flow two times 20 μL of the dynein-bead mixture into the chamber and then seal the chamber with vacuum grease.
7. Mount the slide chamber onto the microscope stage, trap a bead, and place the bead over a surface-bound straight MT, while the stage holding the slide chamber is swept along the

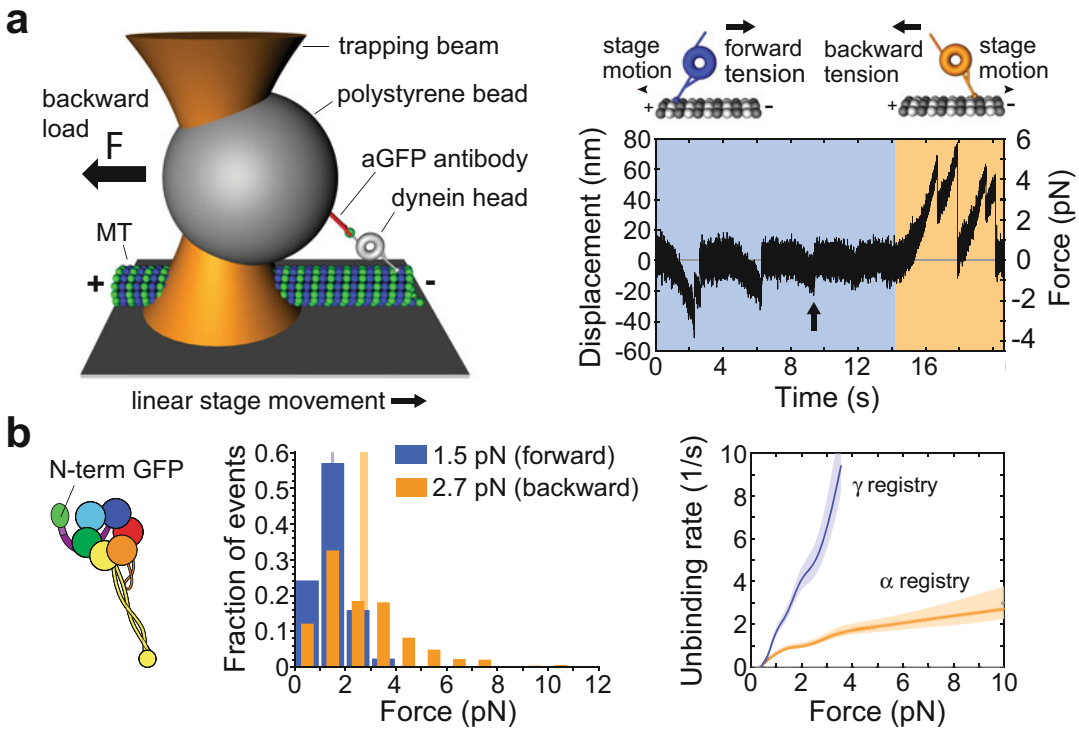

Fig. 3 Dynein-MT bond anisotropy measured with optical tweezers. (**a**) (Left) A trapping bead bound to a dynein motor is held in an optical trap as the microscope stage sweeps back and forth parallel to a MT (not to scale). (Right) Position (force) vs. time for wild-type GFP-Dyn1$_{331kDa}$ in the apo state (6.6 units/mL apyrase added, *see* **Note 22**). Orange and blue shaded areas show periods of applied backward and forward tension, respectively (loading rate: 5.6 pN/s; k = 0.075 pN/nm). After the motor binds the MT, it pulls the bead out of the trap. Force on the motor increases until the dynein-MT bond ruptures at the "unbinding force" (arrow), here ~1 pN. (**b**) (Left) Schematic of dynein with GFP fused to the N-terminus of the linker element. (middle) Normalized histograms of primary forward and backward unbinding forces of GFP-Dyn1$_{331kDa}$ in the apo state. The mean values are noted. Tall vertical bands represent 95% CIs of the means (forward: [1.45, 1.54] pN; backward: [2.59, 2.91] pN), which were estimated by bootstrapping 4000 samples. The number of events in the forward and backward directions: 891 and 536. (Right) Unbinding rate vs. force derived from the data above. The shaded areas were 95% CIs for the mean rates, which were also estimated by bootstrapping. Under forward load, the stalk helices of a *S. cerevisiae* dynein MD assume the γ registry in the apo state with intermediate MT-binding strength and the α registry with strong MT-binding strength under backward load [13, 17, 18]

direction parallel to the long MT axis. The speed of movement is adjusted to produce an apparent loading rate of 5.6 pN/s (the true loading rate depends on the compliance of the motor and is smaller than the apparent rate [13]). To ensure measurements at the single-molecule level, experiments are performed at motor concentrations (used for the coating of the trapping beads) at which less than 30% of the beads exhibit interactions with the MT [25, 26].

8. The measured detachment forces (Fig. 3a, right) are then analyzed using a custom-written MATLAB program [17]. Figure 3 depicts the measured unbinding force histogram (Fig. 3b, left) and the calculated force-dependent unbinding rates (Fig. 3b, right) obtained for single-headed *S. cerevisiae* dynein (Dyn_{331kDa}) in the apo state using the C-Trap.

4 Notes

1. The *S. cerevisiae* dynein construct used in this study is a tail-truncated dynein (Dyn_{331kDa}) [16], which cannot bind the intermediate chain, light intermediate chain, and light chains of dynein.
2. Harvest yeast cells during the log phase to obtain the highest dynein expression when the cells are actively dividing.
3. Add stepwise 0.5 mL of ddH_2O until the pellet is fully resuspended.
4. Drip the cell slurry dropwise into a box containing liquid nitrogen to avoid the cell droplets forming large chunks of cells.
5. These purification steps are performed in the cold room unless specified otherwise.
6. DTT, ATP.Mg, pepstatin A, leupeptin, pefabloc SC, and benzamidine should be added to the solution just shortly before each experiment.
7. Test the functionality of the chilled grinder to make sure the blade is not stuck.
8. Fill the tube with ~3 mL cell lysate each. Balance each pair of tubes with a precision of ±0.1 g and place them in the opposite positions in the rotor.
9. Leave ~0.5 mL in each tube to avoid transferring debris near the pellets.
10. Gently invert the bottle of IgG beads several times until beads are completely resuspended. Transfer the bead slurry using a tip-cut 1000-μL pipette tip. Generally, a 10-μL bead slurry is used for 1 mL of lysate.
11. Skip this step if labeling is not required.
12. The use of membrane filters to separate dynein from the beads is not recommended as dynein tends to bind to membrane surfaces.
13. Use 2 μL of MTs (1 mg/mL) for native dynein.

14. **Steps 1–7** are aimed to prepare the aliquots of the tubulin used for polymerization. **Steps 8–15** are aimed to polymerize the polarity-marked MTs.
15. The purpose of this step is to obtain MT seeds with a higher concentration of fluorescently labeled tubulin.
16. The purpose of this step is to polymerize dim MT extensions from the seeds that are significantly less bright, yielding a bright seed (with the minus end) with a dim extension (with the plus end).
17. The polarity-marked MTs will lose their polarity slowly because of a slow dissociation of tubulin subunits from the MT minus end. Use the MTs within 48 h to ensure that enough polarity-market MTs can be observed on the microscope.
18. The α-casein and Pluronic F-127 are both good blocking reagents for coverslips. The biotinylated α-casein in the solution helps to maintain a constant density of the biotinylated α-casein on the surface. With α-casein instead of biotinylated α-casein in solution, the cover slip-bound biotinylated α-casein would be replaced over time by α-casein, resulting in a decrease of the surface density of biotinylated α-casein.
19. MTs tend to sediment to the bottom of the tube over time. Resuspend the MTs carefully before usage.
20. An immediate wash step helps aligning the MTs with the flow direction.
21. The motility buffer should be prepared fresh. Supply HME30 with α-casein and TCEP immediately before each experiment.
22. The mixture of glucose, glucose oxidase, and catalase forms an oxygen scavenging system, which enzymatically removes oxygen in solution. The added apyrase removes residual ATP for the apo state experiments.

Acknowledgments

X. Liu, L. Rao, and A. Gennerich are supported by the National Institutes of Health grant R01GM098469.

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