Chapter 4

Probing the Force Generation and Stepping Behavior of Cytoplasmic Dynein

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Abstract

Cytoplasmic dynein, which is the largest and arguably the most complex cytoskeletal motor protein, plays fundamental roles during cell division, nuclear positioning, and organelle and mRNA transport, by generating force and movement toward the minus ends of microtubules. Consequently, dynein is central to many physiological processes, and its dysfunction is implicated in human diseases. However, the molecular mechanism by which dynein produces force and movement remains poorly understood. Here, we describe the use of optical tweezers to probe the nanometer-scale motion and force generation of individual dynein molecules, and provide a hands-on protocol for how to purify cytoplasmic dynein from budding yeast in amounts sufficient for single-molecule studies.

Key words: Optical tweezers, Optical trapping, Single-molecule assays, Molecular motors, Cytoplasmic dynein, *Saccharomyces cerevisiae*, Budding yeast, Microtubules

1. Introduction

Despite the increasingly well-characterized in vivo functions of cytoplasmic dynein (1–3), our knowledge of dynein's molecular mechanism is just emerging. A structural and mechanistic model for cytoplasmic dynein is still missing, owing largely to the lack of atomic-resolution structures and the unavailability (until recently) of recombinant dynein capable of processive movement (the ability to take multiple steps) along microtubules (MTs). However, recent single-molecule experiments with purified brain dynein and the generation and characterization of the first recombinant processive dynein have begun to shed light on dynein's molecular mechanism (4–9). In addition, the X-ray structure of dynein's MT-binding domain was recently solved, providing the first structural insights into how dynein binds to its track (10).

Since its application to study individual kinesin (11) and myosin (12) motor proteins, optical trapping microscopy has become an

established and valuable technique in the cytoskeletal motor field for probing the mechanochemistry of force-generating single motors. Optical tweezers can be used to trap and hold a motor-coated polystyrene bead using a near-infrared laser beam that is tightly focused into a diffraction-limited focal volume by an objective lens of high numerical aperture. The bead position can then be tracked with nanometer precision using a quadrant photodiode (QPD) detector or position-sensitive detector (PSD). Once the trapped bead is displaced from the trap center by a molecular motor, a restoring force acts to pull the bead back toward the center of the trap (analogous to the restoring force of a Hookean spring). Modern designs allow the trap to be operated in either a "non-feedback" mode with a fixed trap position, or in a "force-feedback" mode in which the force exerted on the bead is kept constant by maintaining a fixed bead-trap separation using a computer-controlled trap or stage positioning. The nonfeedback mode is commonly used to measure piconewton forces that single cytoskeletal motors generate, by observing motor stepping under increasing opposing loads until movement slows and eventually ceases. The force-feedback mode allows precise measurement of nanometer-scale motor stepping along MTs as a function of constant opposing or assisting forces (8, 13, 14).

Below, we describe the use of an optical tweezers setup to study the force production and force-dependent stepping behavior of individual dynein molecules. We also provide detailed protocols for the use of *Saccharomyces cerevisiae* as a model system to generate recombinant dynein molecules, enabling hypothesis-driven structure–function studies.

2. Materials

2.1. Yeast Growth

1. YPD plates: 10 g Bacto yeast extract, 20 g Bacto peptone, 20 g dextrose, and 20 g agar per 1 L double distilled (dd) H_2O. Autoclave in a 2-L flask for 30 min. Pour plates (100 × 15 mm) when the flask is just cool enough to handle with your bare hands.

2. Liquid YP + sugars: 10 g Bacto yeast extract and 10 g Bacto peptone per 900 ml ddH_2O. Autoclave 30 min. Autoclave 20 g of the sugars (dextrose, raffinose or galactose) separately in 100 ml ddH_2O. Pour the sugar into the YP using sterile technique.

2.2. Dynein Preparation

1. Lysis buffer: For the initial lysis, 4× lysis buffer is used while later steps require 1× lysis buffer. Here, we list the composition of 1× lysis buffer: 30 mM HEPES (pH 7.2), 50 mM K acetate, 2 mM Mg acetate, 1 mM EGTA, 10% glycerol, 1 mM

DTT, 0.1 mM ATP, and 0.5 mM Pefabloc SC (Roche). Note: In all solution preparations, DTT, ATP, and Pefabloc should be freshly added prior to use.

2. 1× wash buffer: 1× lysis buffer supplemented with 250 mM KCl and 0.1% Triton X-100.

3. IgG beads (IgG Sepharose 6 Fast Flow, Amersham). Store at 4°C.

4. BioRad poly-prep column (BioRad Poly-Prep Chromatography Column, BioRad).

5. 1× TEV buffer: 10 mM Tris–HCl (pH 8.0), 150 mM KCl, 10% glycerol, 1 mM DTT, 0.1 mM ATP, and 0.5 mM Pefabloc (Pefabloc does not inhibit TEV protease).

6. Millipore spin column (Millipore).

2.3. Microtubule Binding and Release

1. Tubulin (Cytoskeleton Inc.). Resuspend the lyophilized pellet as described by the manufacturer, aliquot and freeze immediately in liquid nitrogen. Use one aliquot per experiment. Store at −80°C.

2. 1 mM DTT (dithiothreitol) stock solution. Store at −20°C.

3. 10 mM taxol (paclitaxel, Sigma) stock solution dissolved in DMSO. Store at −20°C in small aliquots.

4. 2× tubulin polymerization buffer: 2× BRB80 (see below), 2 mM DTT, 2 mM MgGTP, and 20% DMSO. Store at −80°C in small aliquots.

5. BRB80: 80 mM PIPES (pH 6.8), 2 mM $MgCl_2$, and 1 mM EGTA. Store at room temperature.

6. Apyrase stock solution: 660 U/ml apyrase (Sigma) made in 30 mM HEPES (pH 7.2). Store in aliquots at −20°C.

7. MT-binding buffer: 30 mM HEPES (pH 7.2), 2 mM Mg acetate, 1 mM EGTA, 10% glycerol, 1 mM DTT, 20 μM taxol, and 6.6 U/ml apyrase. Add DTT, taxol and apyrase immediately before use.

8. Sucrose cushion buffer: 30 mM HEPES (pH 7.2), 2 mM Mg acetate, 1 mM EGTA, 10% glycerol, 1 mM DTT, 10 μM taxol, and 40% sucrose.

9. ATP-release buffer: 30 mM HEPES (pH 7.2), 150 mM KCl, 7 mM Mg acetate, 1 mM EGTA, 10% glycerol, 1 mM DTT, 10 μM taxol, and 5 mM ATP (see Note 1).

2.4. Protein Concentration Determination

1. Sypro Red Gel Stain (Sigma).

2. Purified actin protein (Cytoskeleton Inc.). Resuspend the lyophilized pellet as described by the manufacturer, aliquot and freeze immediately in liquid nitrogen. Use one aliquot per experiment.

2.5. Preparation of Biotinylated and TMR-Labeled Microtubules

1. Tubulin (Cytoskeleton Inc.). Resuspend the lyophilized pellet as described by the manufacturer, aliquot and freeze immediately in liquid nitrogen. Use one aliquot per experiment.

2. Biotin-tubulin (Cytoskeleton Inc.). Resuspend the lyophilized pellet as described by the manufacturer, aliquot and freeze immediately in liquid nitrogen. Use one aliquot per experiment.

3. TMR-tubulin (Rhodamine-tubulin, Cytoskeleton Inc.). Resuspend the lyophilized pellet as described by the manufacturer, aliquot and freeze immediately in liquid nitrogen. Use one aliquot per experiment.

4. 2× tubulin polymerization buffer (see above).

5. BRB80 (see above).

6. 10 mM taxol stock solution (see above).

7. 1 M DTT stock solution (see above).

2.6. Coating Polystyrene Beads with GFP Antibodies

1. Trapping beads: 100 mg/ml carboxyl-functionalized microspheres (0.92 μm diameter, Bangs Laboratories). Store at 4°C.

2. Activation buffer: 100 mM NaCl and 10 mM MES (pH 6.0). Store at room temperature.

3. Cross-linking reagents: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, hydrochloride (EDAC, Invitrogen) and N-hydroxysulfosuccinimide (NHSS, Invitrogen). Make sure both reagents are fresh and frozen at −20°C.

4. Quenching reagent: 14.3 M β-mercaptoethanol. Store at room temperature.

5. Coupling buffer: 100 mM sodium phosphate buffer (pH 7.4) prepared by combining 77.4 ml 1 M Na_2HPO_4 and 22.6 ml 1 M NaH_2PO_4. Store at room temperature.

6. Protein solution: 100 mg/ml BSA. Store at 4°C.

7. Antibody solution: 1–4 mg/ml GFP antibody stock solution. Store at −20°C.

8. PBS rinse solution: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4.

9. Quenching solution: 30 mM hydroxylamine hydrochloride ($NH_2OH \cdot HCl$) in PBS (pH 8.0), prepared by dissolving 0.42 g $NH_2OH \cdot HCl$ in 200 ml PBS and adjusting pH to 8.0.

10. Antibacterial reagent: 10% w/v sodium azide (NaN_3) solution.

2.7. Optical Trapping Assay

1. Slide chamber: acid-washed coverslips (15) from Corning (18 × 18 × 0.16 mm), glass microscope slides from Fisher (76.2 × 25.4 × 1 mm), and double-sided sticky tape.

2. Assay buffer: 30 mM HEPES (pH 7.2), 2 mM MgAcetate, and 1 mM EGTA. Filter and store at room temperature.

3. Stock solutions: 100 mM ATP (store at $-80°C$), 25 mg/ml casein (store at $-80°C$), 1 M DTT (store at $-20°C$), 450 mg/ml glucose (2.25 M, store at $-80°C$), 200 mM phosphoenylpyruvate (store at $-20°C$), and 10 mg/ml pyruvate kinase (store at $4°C$).

4. Oxygen-scavenging system: 25 mg/ml glucose oxidase and 3 mg/ml catalase made in assay buffer. Centrifuge the solution at maximum speed in a microfuge for 5 min at $4°C$, filter (Millipore), and store at $4°C$ (do not freeze). Use within a few days.

3. Methods

Cytoplasmic dynein generates forces and motion in eukaryotic cells. To dissect the structural basis of dynein's force-generation and processive motion, we have recently developed methods to engineer and purify cytoplasmic dynein from *S. cerevisiae* (7). In this chapter, we describe in detail the use of *S. cerevisiae* to express and purify a fully functional "minimal" dynein motor (GST-Dyn1$_{331\ kDa}$ (7)). This tail-truncated, artificially dimerized dynein motor retains near normal force- and motion-generating capabilities and can be produced in quantities sufficient for biochemistry and single-molecule studies (7, 8) (Fig. 1a). By performing homologous recombination-based genetic manipulations, this *S. cerevisiae* minimal dynein motor can be used for mutagenesis and structure–function studies, as well as for generating dynein motors with site-specific protein tags for fluorescence labeling.

Ultimately, probing the force-dependent motility of single dynein molecules requires a single-molecule interrogation instrument that can simultaneously measure nanoscale displacements and piconewton forces. This can be achieved with a force-feedback enhanced optical trapping microscope. Here, we first provide a detailed protocol for expressing and purifying recombinant cytoplasmic dynein. We then describe hands-on protocols for utilizing a custom-built force-feedback optical trapping microscope to determine the stall force, force–velocity relationship and the nanoscale stepping behavior of single dynein molecules.

3.1. Growth and Harvesting of Minimal Dynein-Expressing S. cerevisiae Strains

This protocol is for expression of minimal processive dynein-containing strains. The minimal processive dynein motor is a homodimer of two 331 kDa dynein monomers (amino acids 1219–4093 of the *S. cerevisiae* Dyn1 protein) that have been artificially dimerized with glutathione S-transferase (GST)
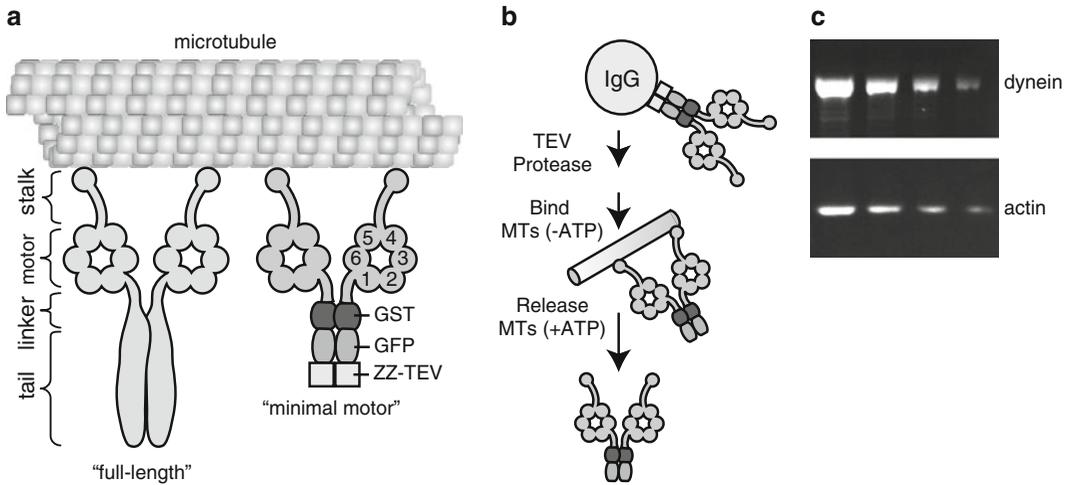
Fig. 1. Purification of GST-Dyn1$_{331 \text{ kDa}}$ from *S. cerevisiae*. (**a**) Full-length cytoplasmic dynein is a dimer of two 470-kDa heavy chains. To make the minimal dimer (GST-Dyn1$_{331 \text{ kDa}}$), the tail of full-length dynein was truncated and then artificially dimerized with GST [7]. This construct also contains an amino terminal purification tag (ZZ-TEV) and GFP for bead attachment for optical trapping experiments. (**b**) An overview of the purification protocol for cytoplasmic dynein. After cell lysis and an ultracentrifugation step, dynein is bound to IgG beads via the ZZ tag. After washing the beads, dynein is cleaved from the IgG beads with TEV protease. As a second affinity purification step, dynein is bound to MTs in the absence of MgATP and then released from MTs in the presence of MgATP. (**c**) SDS-PAGE gel stained with Sypro-Red (Sigma) of purified minimal dynein. Serial dilutions of dynein (*top*) and the actin standard (*bottom*) are shown.

(Fig. 1a) (7). High expression levels are achieved by using a galactose-inducible promoter to drive dynein expression. A list of published strains that express variants of the minimal dimer can be found in Table 1. It is also possible to purify full-length native yeast dynein (7, 16). However, yields for the full-length protein are 50–100 times lower compared to the minimal dimer and the protein is more difficult to purify. Thus, for the purpose of this book chapter, we have chosen to focus on the purification protocol used for the minimal processive dynein.

1. From a frozen glycerol stock, streak yeast cells onto a YPD plate and incubate the plate at 30°C for 2–3 days.

2. Inoculate 10 ml of liquid YPD medium with a single yeast colony and grow in a 25-ml test tube overnight, shaking (250 rpm) at 30°C.

3. The following morning, add the entire 10 ml YPD culture to 50 ml YP + 2% raffinose in a 250-ml Erlenmeyer flask. Grow by shaking (250 rpm) at 30°C for the rest of the day.

4. At the end of the day, transfer the 50 ml YP + raffinose yeast culture to 2 L YP + 2% galactose in a 6-L Erlenmeyer flask. Grow by shaking (200 rpm) at 30°C overnight until the OD$_{600}$ reaches 1.5–2.0 (approximately 16–22 h).

Table 1
Yeast strains expressing minimal processive dynein motors

Dynein description	Strain name	Genotype	Reference
Minimal dimer	VY149	*pGAL-ZZ-TEV-GFP-3XHA-GST-331DYN1*	(7)
Minimal dimer with a C-terminal HaloTag	VY208	*pGAL-ZZ-TEV-GFP-3XHA-GST-331DYN1-gs-DHA1-KanR*	(7)
Minimal dimer with a N-terminal HaloTag	VY268	*PAC11-13MYC::TRP; ZZ-TEV-gs-DHA-GST-331D*	(7)
Minimal dimer with a point mutation in AAA domain 1 (E1849Q)	RPY546	*pGAL-ZZ-TEV-GFP-3XHA-GST-D6-DYN1-E1849Q-gs-DHA-KanR*	(9)
Minimal dimer with a point mutation in AAA domain 3 (E2488Q)	RPY542	*pGAL-ZZ-TEV-GFP-3XHA-GST-D6-DYN1-AAA3E2488Q-gs-DHA-KanR*	(9)
Minimal dimer with a point mutation in AAA domain 4 (E2819Q)	RPY548	*pGAL-ZZ-TEV-GFP-3XHA-GST-D6-DYN1-E2819Q-gs-DHA-KanR*	(9)

These yeast strains all express minimal dynein (the C-terminal 331 kDa of the protein) that is dimerized with GST. All strains are made in a parent strain with the following genotype: *MATa; his3-1,15; ura3-1; leu2-3,112; ade2-1; trp1-1; can1-100; pep4Δ::HIS5; prb1Δ*. "DHA" refers to the HaloTag (Promega) and "gs" refers to glycine and serine amino acids used as spacers. The N-terminal tag includes the galactose promoter (pGAL), two copies of the IgG-binding motif (ZZ), a TEV protease cleavage site (TEV), green-fluorescent protein (GFP), three copies of the HA-epitope tag (HA), and GST for dimerization (GST)

5. To harvest the cells, centrifuge the culture in 1-L Beckman bottles (Beckman) at 6,000 rpm ($6,900 \times g$) at 4°C in a Beckman JLA 8.1 rotor in an Avanti J-26 XP Beckman centrifuge or equivalent.

6. Decant the supernatant and resuspend the cell pellets completely in 1 L ddH$_2$O. Centrifuge again as in step 5.

7. Decant the water and resuspend the cell pellet in any residual ddH$_2$O that remains in the bottle using a 10-ml pipette (it may be necessary to add 1–2 ml of ddH$_2$O).

8. Using a 10-ml pipette, drop-freeze the cell slurry into liquid nitrogen. The frozen yeast bears some resemblance to popcorn at this stage. From this point on the yeast pellets should not be allowed to thaw. Store the frozen yeast pellets at −80°C until ready to proceed.

3.2. Purification of S. cerevisiae Cytoplasmic Dynein

All minimal dynein dimer constructs that we have made contain an amino-terminal (dynein tail) purification tag consisting of two copies of an IgG-binding domain (ZZ) and a TEV protease cleavage site (Fig. 1a, b).

1. Lyse the frozen yeast pellets by grinding in a coffee grinder (we use KitchenAid BCG100 Blade Coffee Grinders). It is important to keep the yeast pellets frozen at all times. The coffee grinder must be pre-chilled with liquid nitrogen and it may be necessary to add liquid nitrogen a second time during the grinding. The yeast pellets should be ground until they resemble powdered sugar or a very fine espresso grind (approximately 1 min of total grinding time). Note: to prevent loss of yeast pellets do not operate the coffee grinder until all liquid nitrogen has evaporated. Wear heavy gloves while operating the coffee grinder. Alternatively, the frozen yeast pellets can by lysed by grinding manually using a mortar and pestle in the presence of liquid nitrogen.

2. Remove the yeast powder from the coffee grinder with a small spatula to a 50-ml conical tube. Immediately add 4× lysis buffer to the yeast powder so that the final concentration of lysis buffer after the yeast powder thaws is 1× (approximately 1 ml 4× lysis buffer per 10 ml yeast powder).

3. Place the conical tube into a 37°C water bath to quickly thaw the yeast powder. As soon as the powder has thawed, the lysate should be placed on ice. All future steps should be performed on ice or in a cold room unless otherwise specified.

4. Estimate the final lysis volume and adjust the lysis buffer concentration to 1× (using 4× lysis buffer) if necessary.

5. To remove cell debris and unlysed cells, centrifuge the lysate in a Beckman TLA110 rotor at 90,000 rpm (338,000 × g) for 20 min at 4°C in a Beckman tabletop ultracentrifuge. Use thick walled polycarbonate tubes (Beckman). Tubes must be balanced to 0.01 g prior to centrifugation.

6. During the spin, prepare IgG beads for the affinity purification step. Use 10 μl of 50% bead slurry per 1 ml of lysate (volume estimated in step 4). Wash the beads twice with at least 10× the bead volume in a BioRad Poly-Prep Column.

7. Remove the supernatant from the spin in step 5. Avoid the lipid layer that is floating on top of the supernatant. Pipette the supernatant into a 15- or 50-ml conical tube. Remove and freeze (in liquid nitrogen) 20 μl for a gel sample.

8. Add the washed beads to the extract (use the extract to pipette the beads out of the BioRad column). Incubate the beads and extract in the cold room for 1–2 h on a rocking platform at a slow speed to avoid air bubble production (which can denature proteins).

9. Wash the beads twice (20× bead volume per wash) with 1× wash buffer.

10. Wash the beads once (20× bead volume) with 1× TEV buffer.

11. Optional: Most of the minimal dynein constructs we have made include a HaloTag (Promega) on either the N or C terminus. To label the HaloTag with a fluorophore or biotin, add 100 μl 1× TEV buffer per 100 μl bead bed volume. The labeling step can be done directly on the BioRad column (be sure to cap the bottom of the column). Add HaloTag ligand to a final concentration of 10 μM and incubate at room temperature for 10 min (covered with foil if using a fluorophore ligand).

12. Optional: If labeling the HaloTag, wash the beads an additional two times (20× bead volume per wash) with 1× TEV buffer to remove any unbound dye or biotin.

13. Cleave dynein off the beads with TEV protease. Transfer the beads from the BioRad column to a microfuge tube with 100 μl 1× TEV buffer per 100 μl bead bed volume (depending on the pipette tips used, it may be necessary to cut the tip of the pipette with a razor blade to ensure the beads can be removed). Note: the volume added at this step will be equal to the final elution volume. Add TEV protease (see Note 2). We use 4 μg TEV protease per 100 μl TEV buffer added in the previous step. Incubate at 16°C for 1–2 h (or 4°C overnight) rocking slowly (avoid air bubble formation).

14. Transfer the entire reaction (beads and buffer containing cleaved dynein) from the microfuge tube to a 2-ml Millipore spin column. Centrifuge the spin column in a microfuge at maximum speed at 4°C for 30 s. Save the beads for a gel sample.

15. Prepare 50 μl aliquots of the supernatant and freeze immediately in liquid nitrogen. Store the protein at −80°C. Alternatively, you can proceed directly to the MT-binding and -release step without freezing the protein in between.

3.3. Microtubule Binding and Release

As a second affinity purification step that also removes any non-functional motors, dynein is mixed with MTs in the absence of ATP (tight MT-binding state) and then released from MTs in the presence of ATP (weak MT-binding state). The first steps of this protocol describe how to polymerize tubulin, while the later steps include the MT binding and release of dynein.

1. Thaw one aliquot of tubulin and one aliquot of DTT and place both on ice. Thaw one aliquot of taxol and keep at room temperature (see Note 3).

2. Polymerize 15 μl 100 μM tubulin by mixing with 15 μl of 2× polymerization buffer. Incubate at 37°C for 20 min. This will yield 30 μl of 50 μM MTs. From this point on, the MTs

should never be placed on ice and taxol should be included in all subsequent steps to prevent MT depolymerization.

3. Stabilize MTs with taxol by combining the MTs (30 μl of 50 μM MTs) from the previous step with 30 μl BRB80 supplemented with 1 mM DTT and 100 μM taxol. Incubate at 37°C for 10 min. The final yield will be ~60 μl of 25 μM MTs.

4. MT-binding step: thaw a 50 μl aliquot of dynein solution (~0.6 μM) and place on ice. Mix together 50 μl dynein solution, 25 μl MTs (~25 μM), and 25 μl MT-binding buffer. Incubate at room temperature for 10 min. Gently pipette the 100 μl reaction mixture over 100 μl sucrose cushion buffer and centrifuge in a Beckman TLA100 rotor (the rotor should be at room temperature) for 15 min at 50,000 rpm (109,000 × g) at 22°C.

5. After the spin, remove the top 50 μl of the supernatant for a gel sample. Discard the remainder of the supernatant and wash the MT pellet carefully with 100 μl MT-binding buffer, being careful not to disrupt the dynein-MT pellet.

6. Resuspend the MT pellet in 100 μl ATP-release buffer and incubate at room temperature for 10 min.

7. Centrifuge the resuspended pellet in a Beckman TLA100 rotor (the rotor should be at room temperature) for 15 min at 50,000 rpm (109,000 × g) at 22°C.

8. Remove the supernatant (dynein-containing solution), aliquot (3–5 μl samples, see Note 4) and freeze immediately in liquid nitrogen. Store the protein at −80°C.

3.4. Protein Quantification

We quantify dynein protein concentration by running a SDS-PAGE gel with a dilution series of dynein and a dilution series of a protein of known concentration (e.g., actin, Fig. 1c). The gel is then stained with Sypro Red Gel Stain. Sypro Red's fluorescence intensity is linear with protein quantity over several orders of magnitude. After staining, densitometry analysis is used to quantify the intensity of the known actin concentrations and a standard curve is generated. The intensity of the dynein bands is also quantified and the actin standard curve is used to assign protein concentration to the dynein sample. The typical concentration of minimal dimeric dynein motor from a 2 L preparation (eluted in 300 μl) before the MT-binding and -release step is 500 μg/ml or ~0.6 μM.

3.5. Preparation of Biotinylated and TMR-Labeled Microtubules

In this step, unlabelled tubulin is polymerized in the presence of biotinylated and fluorescently labeled tubulin subunits. The biotin is used for attachment to coverslips and the fluorescent label for visualization. We purify tubulin from bovine or porcine brains using

the protocol from the Mitchison lab website: http://mitchison. med.harvard.edu/protocols/tubprep.html. Alternatively, tubulin can be obtained from Cytoskeleton Inc.

1. Spin unlabeled tubulin in a Beckman TLA100 rotor at 90,000 rpm (352,000 × g) for 10 min at 4°C in thick-walled polycarbonate tubes (Beckman) to remove any tubulin aggregates. Keep the tubulin on ice at this point.

2. Mix together 18 μl 50 μM unlabelled tubulin, 1 μl 100 μM biotin-tubulin and 1 μl 100 μM TMR-tubulin. Mix gently and incubate on ice (covered with foil) for 10 min.

3. Add 20 μl 2× polymerization mix to the tubulin prepared in step 2. Mix gently and incubate in the dark at 37°C for 30 min. The tubulin will polymerize during this step. From this point on the MTs should never be placed on ice, as this will depolymerize the MTs.

4. Add 40 μl BRB80 supplemented with 1 mM DTT and 20 μM taxol and incubate at 37°C for 10 min.

5. Store the taxol-stabilized MTs in the dark at room temperature. Polymerized MTs are stable for several days.

3.6. Coating Polystyrene Beads with GFP Antibodies

1. Wash carboxyl-modified microspheres in activation buffer. Pipette 100 μl bead solution into a 1.5-ml microfuge tube and add 0.9 ml activation buffer. Centrifuge 15 min at 2,000 × g (all bead centrifugation steps should be performed at 2,000 × g) and discard the supernatant. Resuspend in 1 ml activation buffer (vortex and sonicate solution for a few seconds). Repeat this procedure 2–3 times.

2. Following the final resuspension step, add 10 mg of both EDAC and NHSS. Sonicate the solution for 10 s in a water bath sonicator and incubate on a rocking platform at low speed at room temperature for 30 min.

3. Add 1.4 μl 14.3 M β-mercaptoethanol to quench the EDAC reaction.

4. Wash the beads four times in coupling buffer (centrifuge the solution, discard the supernatant and resuspend the beads in coupling buffer). Ensure that the beads are well suspended before the final centrifugation step (vortex and sonicate for a few seconds).

5. Mix the antibody solution of your choice with BSA to bring the final total protein concentration to 5–10 mg/ml (500–1,000 μl volume) (see Note 5). We use approximately 0.3 mg of GFP antibody. React 10 mg of beads with the antibody-BSA solution for 2–4 h at room temperature (or overnight at 4°C) with constant mixing (sonicate briefly if necessary in a water bath sonicator).

6. Wash the beads once in PBS rinse solution and resuspend in quenching solution. Incubate on a rocking platform at room temperature for 30 min (or overnight at 4°C).

7. Rinse beads four times in PBS. Resuspend beads in 100 μl PBS (original volume of bead solution) containing 0.1% NaN$_3$ and 0.5 mg/ml BSA. Store the bead stock solution at 4°C (do not freeze the beads).

3.7. Optical Trapping Assay

1. Make a flow cell by placing two strips of double-sided sticky tape between a standard microscope slide and a 160-μm thick 18 mm × 18 mm coverslip to form a channel (Fig. 2a). If the two pieces of tape are about 3 mm apart, the approximate volume of the flow cell will be 10 μl.

2. Dilute 2 μl bead stock solution (resuspend beads before use by pipetting, but do not sonicate) with 18 μl assay buffer. This solution should be stored at 4°C and can be kept for a few days. Sonicate the diluted solution in a water bath sonicator for 10 s before use.

3. Thaw one aliquot of each: ATP (100 mM), casein (25 mg/ml), DTT (1 M), and glucose (2.25 M), and store on ice.
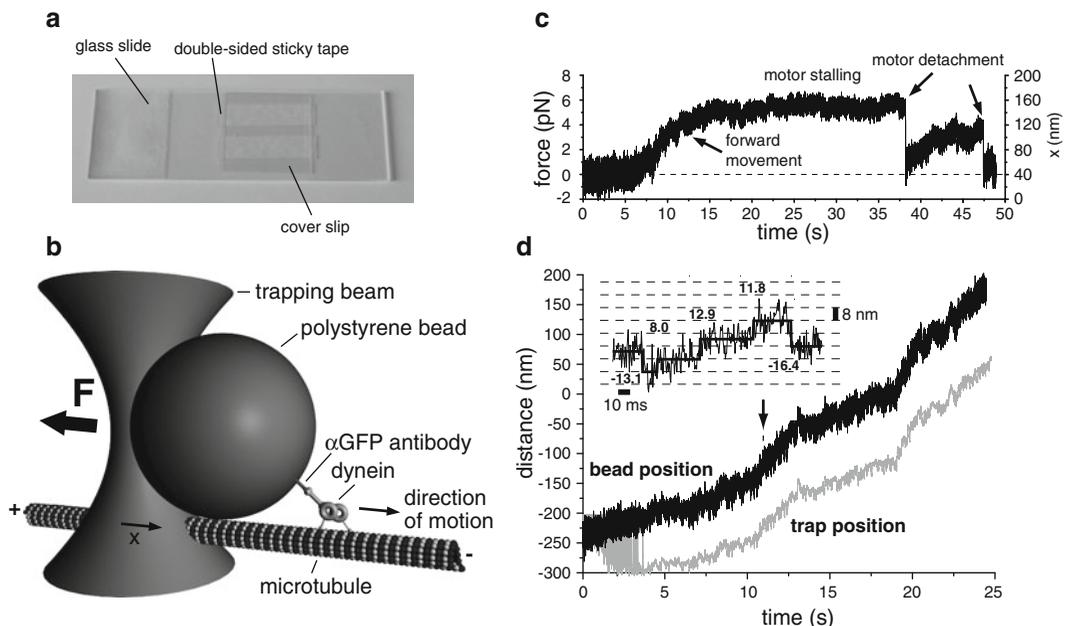
Fig. 2. Force generation and stepping of GST-Dyn1$_{331\ kDa}$. (**a**) Custom-built flow cell. (**b**) Optical trapping assay (not to scale). (**c**) Force generation of a single GST-Dyn1$_{331\ kDa}$ dynein molecule at 1 mM ATP in a fixed optical trap (nonfeedback mode). (**d**) Processive forward stepping of a single dynein molecule against a constant load of 4 pN (force-feedback mode). The bead position and trap position are shown in *black* and *gray*, respectively (trap stiffness: $k = 0.039$ pN/nm). The inset shows an example trace segment with detected steps. The segment corresponds to the part of the displacement trace indicated by the *black bar* (*arrow*).

4. Quickly thaw a 3 µl aliquot of frozen dynein (from Subheading 3.3, step 8) and place on ice immediately. Dilute the motor with assay buffer containing 1 mg/ml casein. Start with an initial dilution of 1,000× by serially diluting the motor in tenfold dilution steps.

5. Combine 4 µl diluted bead solution (from step 2) and 4 µl motor solution (from step 4) and incubate on ice for 10 min.

6. During the incubation time, coat the flow cell with biotinylated BSA for 2 min, incubate with streptavidin for 2 min, and then incubate with biotinylated and TMR-labeled MTs for 5 min. Finally, wash the flow cell with 20 µl assay buffer + 1 mg/ml casein.

7. Prepare motility buffer: 1 µl 100 mM ATP, 4 µl 25 mg/ml casein, 1 µl 1 M DTT, 1 µl 2.25 M glucose, and 2 µl assay buffer, and place on ice. Add 1 µl oxygen-scavenging solution just prior to adding the motility buffer to the bead-dynein solution in the next step.

8. Combine motility buffer with the bead-dynein solution: mix 4 µl prepared motility buffer (step 7), 28 µl assay buffer, and 8 µl bead-dynein solution (step 5). The final solution (40 µl) contains 1 mM MgATP, 1 mg/ml casein, 10 mM DTT, 22.5 mM glucose, 0.25 mg/ml glucose oxidase, and 0.03 mg/ml catalase in assay buffer (30 mM HEPES (pH 7.2), 2 mM Mg acetate, and 1 mM EGTA).

9. Finally, pipette and flow the solution into the slide chamber and seal the flow cell with clear nail polish.

3.8. Measuring Dynein's Stall Force

1. Mount the slide chamber to the microscope stage and adjust the microscope condenser using a sufficient amount of immersion oil.

2. Turn on all required components for the fluorescence-imaging system (see Note 6) and adjust the z-position of the stage to bring the surface-attached MTs into the object plane.

3. Select a horizontally aligned MT (look for straight MTs) and position it (using the x-/y-stage positioning system) near the center of the object field. Shutter the fluorescence excitation light to prevent further photo bleaching.

4. Trap a diffusing bead (see Note 6) and measure the response of the photo detector by scanning the trapped bead along the x-axis of the object field (from −200 to +200 nm). Use a MatLab script (or equivalent) to convert the QPD-voltage signals into nm-displacements.

5. Calibrate the trap stiffness by measuring the amplitude of thermal diffusion (equipartition analysis) and/or by analyzing the power spectrum of thermal diffusion (see Note 7).

6. Open the shutter that blocks the fluorescence excitation light and position the axial center of the preselected MT (see step 3) in the center of the object field, which coincides with the center of the QPD detection system.

7. Move the MT near the trapped bead by adjusting the *z*-position of the stage. The thermal noise of the bead will decrease once the MT makes contact with the trapped bead. Keep the MT surface as close to the trapped bead as possible, but avoid direct contact (Fig. 2b).

8. Observe the bead position for 1 min. The positional fluctuations of the trapped bead will decrease once a dynein motor binds to the MT. Upon MT binding, the motor will advance along the MT and pull the bead away from the fixed trap center (in the nonfeedback mode) (Fig. 2c). If MT binding and subsequent bead movement is not observed, reposition the bead on the MT and monitor for an additional minute.

9. To ensure that the behavior of single molecules is being measured, adjust the motor dilution such that the number of beads binding to or moving along MTs is ≤0.3. At this dynein-to-bead ratio, the probability that two or more motors generate the bead movement is <0.01 (8) (see Note 8).

10. Determine the dynein stall force using the established dynein-to-bead ratio. The force load (F) that the motor experiences increases with increasing bead-trap separation x ($F = -k \cdot x$). The motor movement will gradually slow down and eventually cease once the stall force is reached (Fig. 2c). A stalling event can be identified as a horizontal plateau in the displacement trace. It is important to note that premature dissociation of dynein from the MT can also terminate a run before the stall force is achieved (Fig. 2c, right). To avoid including premature dissociation events in the stall force measurements, include only stalls in the analysis that last for more than 10 s (see Note 9). To calculate the stall force, multiply the trap stiffness by the mean maximum distance reached. We typically accumulate ~100 stall-force values to create a stall-force histogram (8).

3.9. Measuring Dynein's Force–Velocity Relationship

1. Prepare a sealed flow chamber with dynein-coated beads and 1 mM ATP (saturating concentration) as described in Subheading 3.7.

2. Record dynein displacement traces under varying average constant force loads using the force-feedback mode of the optical trap (Fig. 2d). To minimize force errors associated with Brownian movements of the bead, adjust the trap stiffness so that the bead-trap separation is ~100 nm at the desired load (17). For example, set the trap stiffness to 0.04 pN/nm

when acquiring displacement traces under 4 pN constant backward load (Fig. 2d). At the smallest force load of 0.5 pN, set the trap stiffness to 0.01 pN/nm, which corresponds to a bead-trap separation of 50 nm.

3. Calculate motor velocity by fitting the displacement traces of the beads moving under constant load with a line and determine its slope.

3.10. Measuring Dynein's Nanoscale Motion as a Function of Load and ATP

1. Perform force-feedback measurements at various constant force loads as described above using saturating (1 mM) and subsaturating ATP concentrations. In the case of yeast dynein, motor stepping is slow enough to be resolved even at saturating ATP concentrations (Fig. 2d, inset) (7). The K_m (the ATP concentration at which motor velocity reaches half maximal velocity) of *S. cerevisiae* cytoplasmic dynein is approximately 10 μM (8). When working with ATP concentrations in the micromolar range, use an ATP regeneration system by adding 1 μl 200 mM phosphoenylpyruvate and 1 μl 10 mg/ml pyruvate kinase in place of the 2 μl assay buffer when preparing the motility buffer (see Subheading 3.7, step 7).

2. Motor steps (approximate center-of-mass movement of dynein) can be determined from the bead displacement records using the step-finding algorithm developed by Kerssemakers et al. (18) (Fig. 2d, inset) (see Note 10).

4. Notes

1. In all solutions containing ATP, Mg^{++} should be present at an equal or greater concentration than the ATP concentration. This is because Mg^{++} ions are important for coordinating the binding of ATP to dynein's ATP-binding sites.

2. We express and purify TEV protease using the method of Kapust et al. (19). TEV protease can also be purchased from Invitrogen.

3. Because the taxol stock solution is made in 100% DMSO, it will freeze immediately on ice. The taxol stock can be kept at room temperature during the MT-binding and -release step.

4. After the MT-binding and -release step, we aliquot and freeze the dynein solution into small (3–5 μl) aliquots and do not freeze/thaw the protein again. Each aliquot is intended for a single experiment (one slide chamber). Because dynein motors become nonfunctional more rapidly at 4°C than when frozen at −80°C, this ensures that each experiment is performed at the same concentration of functional protein.

5. The surface-functionalized beads will aggregate when the local protein concentration is not high enough to comprise a monolayer on the bead surface. Resuspending the beads in a premixed BSA-antibody solution ensures a complete surface coating of the beads.

6. Measurements are performed at room temperature ($25 \pm 0.5°C$) with a custom-built, force-feedback optical trapping microscope. A near-infrared laser beam (Nd:YVO$_4$, 1,064 nm; Spectra Physics) is coupled to an inverted microscope equipped with a $63\times/1.4$ NA oil-immersion objective (Plan-Apochromat, Carl Zeiss, Thornwood, NY). To generate two independently controllable traps, the laser beam is split into horizontally (trap 1) and vertically (trap 2) polarized light. Trap 1 is steered by a computer-controlled mirror system and is used to capture a bead anywhere in the object field. Trap 1 then transfers the captured bead to trap 2 in the object center. The position of trap 2 is restricted to a 1.5×1.5 μm area located in the center of the object field and is integrated into a position feedback loop that is controlled by a DSP-board (M67, Innovative Integration, Simi Valley, CA) via a two-axis acousto-optic deflection system (DTD-274HA6, IntraAction, Bellwood, IL). All optical trapping experiments are performed with trap 2. Force-feedback is operational in an area of ±200 nm along both the x- and y-axis of the object field. The microscope is further equipped with a Xenon lamp (Oriel, Stamford, CT) for bright field and epifluorescence imaging, as well as a computer-controlled x-/y-stage. The brightfield image is captured by both a charge-coupled camera (CCD) and a QPD detector, whereas the fluorescence image is captured by a silicon intensified (SIT) camera, allowing visualization of the TMR-labeled MTs. The position of the trapped bead detected by the QPD is recorded at 2 kHz and filtered at 1 kHz.

7. According to the equipartition theorem, the energy associated with the fluctuations (with respect to the x-axis) of the trapped bead $0.5k \cdot <x^2>$ is equal to $0.5k_B T$ of thermal energy, where $<x^2>$ is the positional variance, k is the trap stiffness, k_B is the Boltzmann constant, and T is the absolute temperature, respectively. This yields an equation for the trap stiffness k: $k = k_B T/<x^2>$ (for a detailed discussion on bead calibration methods, see ref. [20]).

8. To determine if a motor is processive, it is customary and recommended (especially in the case of an uncharacterized motor) to determine the fraction of motor-coated beads binding to and moving along filaments as a function of the relative motor concentration. In these experiments, the bead concentration is kept constant for all measurements while the motor concentration is

varied. If the resulting data fits well with the Poisson probability $1 - \exp(-\lambda C)$ that a given bead carries one or more motor molecules (where C is the relative motor concentration and λ is a fit parameter; (21)), the data provide evidence that a single motor is sufficient to drive continuous bead movement (as is the case for cytoplasmic dynein). However, if the data can be better fit by the probability $1 - \exp(-\lambda C) - (\lambda C)\exp(-\lambda C)$, this suggests that bead movement requires two or more motors, and is indicative of a nonprocessive motor.

9. *S. cerevisiae* cytoplasmic dynein typically stalls for several minutes before it detaches from its track (8), whereas mammalian cytoplasmic dynein tends to detach prematurely or within a few seconds after motor stalling (4, 5).

10. This algorithm assumes that steps are hidden in normal-distributed noise but makes no assumptions about step size or duration. The only parameter that is user-supplied is an estimate of the number of hidden steps. This parameter is chosen so that the algorithm appears to slightly "overfit" the data (18). The resulting data are then manually examined and only those steps that can be visually separated from noise are included in step size histograms. Using this method and depending on the applied load, one can typically assign step sizes to 40–90% of a given dynein run.

Acknowledgments

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