Chapter 4

Insect Cell-Based Expression of Cytoskeletal Motor Proteins for Single-Molecule Studies

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Abstract

Cytoskeletal motor proteins are essential molecular machines that hydrolyze ATP to generate force and motion along cytoskeletal filaments. Members of the dynein and kinesin superfamilies play critical roles in transporting biological payloads (such as proteins, organelles, and vesicles) along microtubule pathways, cause the beating of flagella and cilia, and act within the mitotic and meiotic spindles to segregate replicated chromosomes to progeny cells. Understanding the underlying mechanisms and behaviors of motor proteins is critical to provide better strategies for the treatment of motor protein-related diseases. Here, we provide detailed protocols for the recombinant expression of the Kinesin-1 motor KIF5C using a baculovirus/insect cell system and provide updated protocols for performing single-molecule studies using total internal reflection fluorescence microscopy and optical tweezers to study the motility and force generation of the purified motor.

Key words Single molecule, Optical tweezers, Fluorescence, Microtubules, Motor proteins, Kinesin, KIF5A, Insect cells, Baculovirus, Plasmid

1 Introduction

Cytoskeletal motor proteins, which comprise myosin, kinesin, and cytoplasmic dynein, are an important class of molecular machines that convert chemical energy into mechanical motion along cytoskeletal filaments [1, 2]. Members of the dynein and kinesin superfamilies play critical roles in transporting a diverse set of cargos, such as protein complexes, signaling molecules, organelles, and vesicles, along microtubule filaments. They also act within the mitotic spindle to align replicated chromosomes and to separate and move the sister chromatids into the daughter cells [3–7]. Many advanced techniques, such as single-molecule fluorescence microscopy and optical tweezers, combined with structure-function studies, have been employed to determine the mechanisms that underly the function of these motors [8–10]. To date, however, the recombinant expression of fully active cytoskeletal motors, especially

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MDa-sized mammalian cytoskeletal motor proteins, remains a major challenge for in vitro single-molecule studies.

Since its discovery in 1983, the baculovirus/insect cell expression system has been extensively used for production of recombinant proteins, and a number of technological improvements have been achieved to simplify the expression process and to increase the variety of transfer vectors [11–16]. Compared to the *E.coli*-based expression system, the baculovirus/insect cell expression system allows the high-yield expression of large proteins and simple glycosylation and posttranslational modifications [11, 17–19]. The insect cell expression system has been adopted by research groups worldwide for the recombinant expression of cytoskeletal molecular motors, including myosin motors and full-length human dynein and members of kinesin families, which had proven difficult or impossible to express in other systems [20–23].

In this chapter, we provide simplified and improved protocol for the insect cell-based expression of mammalian motor proteins for single-molecule studies. We begin with the description of the construction of a recombinant bacmid DNA using the site-specific transposition of the gene of interest into a baculovirus shuttle vector (bacmid), placed typically behind the strong polyhedrin promotor. We continue to describe how by selective antibiotics, the bacmid DNA from positive bacterial clones is isolated and used to transfect insect cells and produce recombinant viruses. Subsequently, we describe how the viral stock is used to infect a larger volume of insect cells to generate a higher titer of virus stock. We then describe how the desired protein is recombinantly expressed and purified from infected insect cells using the amplified viral stock (Fig. 1). Finally, we describe how single-molecule fluorescence microscopy and optical tweezers are employed to study the motility and force generation of the purified kinesin-1 motor, KIF5C.

2 Materials

2.1 Bacmid Generation

2.1.1 Bacmid Selection and Plate Preparation

1. Difco™ LB agar (Fisher Scientific, #DF0812-07-1).
2. Petri dish: 60 mm × 15 mm (Fisher Scientific, #35839).
3. Gentamycin: dissolved gentamycin at 10 mg/mL in distilled water and sterilize solution using a 0.22 μm sterile syringe filter, then aliquot and store at −20 °C (Thermo Fisher Scientific, #15710064).
4. Tetracycline: dissolve tetracycline powder in 70% ethanol to a final concentration of 1 mg/mL, sterilize using a 0.22 μm sterile syringe filter, and then aliquot and store at −20 °C (Thermo Fisher Scientific, #J61714.06).

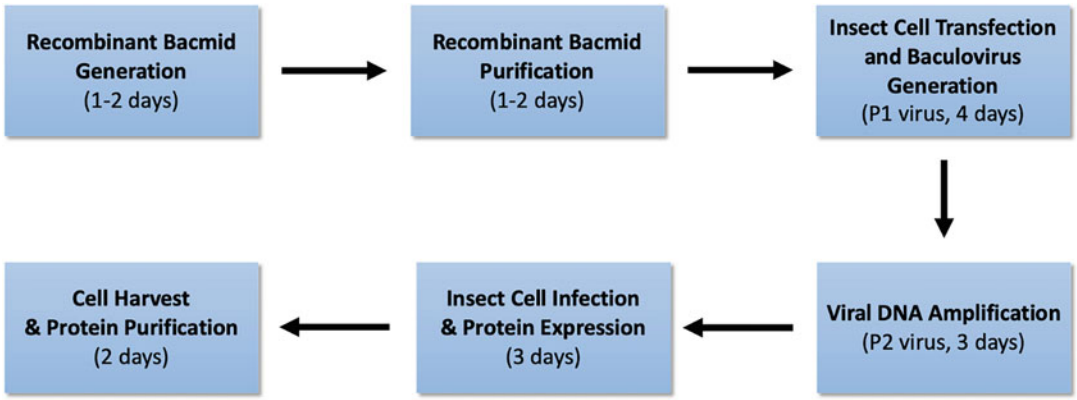

Fig. 1 Processes of recombinant expression of motor proteins using the baculovirus/insect cell system

5. Kanamycin sulfate: dissolve kanamycin sulfate in distilled water to a final concentration of 30 mg/mL, sterilize using a 0.22 μm sterile syringe filter, and then aliquot and store at −20 °C (Thermo Fisher Scientific, #450810100).
6. IPTG: dissolve isopropythlthio-β-galactoside in water to a final concentration of 50 mg/mL, sterilize using a 0.22 μm sterile syringe filter, and then aliquot and store at −20 °C (Thermo Fisher Scientific, #15529019).
7. Bluo-Gal: dissolve halogenated indolyl-β-galactoside in dimethylformamide to a final concentration of 20 mg/mL, sterilize using a 0.22 μm sterile syringe filter, and then aliquot and store at −20 °C (Thermo Fisher Scientific, #15519028).

2.1.2 Recombinant Bacmid Generation

1. Max Efficiency™ DH10Bac competent cells, used to produce recombinant baculoviruses for the expression of eukaryotic proteins and stored at −80 °C (Gibco, #10361012).
2. pFastBac plasmid containing DNA of interest, store at −20 °C.

2.2 Bacmid Purification

2.2.1 Bacmid Culture Preparation

1. Difco™ LB broth: dissolve 10 g LB broth powder to 500 mL distilled water and autoclave to sterilize, then store at room temperature (Fisher Scientific, #DF0446-17-3).
2. Corning™ Falcon round-bottom tube: sterile 14 mL test tube (Fisher Scientific, #14–959-11B).

2.2.2 Bacmid Culture Purification

1. Microcentrifuge tube: 2 mL low protein binding tubes (Thermo Scientific, #PI90410).
2. QIAprep spin miniprep kit for the purification of plasmid DNA with high purity and stored at room temperature (Qiagen, #27106).
3. Puritan™ wood applicator stick, autoclaved to sterilize and stored at room temperature (Fisher Scientific #22029680).

3. Ethanol: ethyl alcohol, diluted with distilled water to a final concentration of 70% and stored at room temperature (Millipore Sigma, #64-17-5).
4. Isopropanol: 2-propanol, store at room temperature (Millipore Sigma, #19516).

2.3 Insect Cell Transfection, Baculovirus Generation, and Protein Expression

1. Sf9 cells: adapted to serum-free suspension culture in Sf-900™II SFM, stored in liquid nitrogen (Thermo Fisher Scientific, #11496015).
2. Sf-900™II SFM: a serum-free and protein-free insect cell culture medium optimized for the growth and maintenance of *Spodoptera frugiperda* (Sf9 and Sf21) cells, stored at 4 °C (Thermo Fisher Scientific, #10902104).
3. A biosafety hood.
4. A non-humidity insect cell incubator.
5. Trypan blue solution: routinely used as a cell stain to assess cell viability, stored at room temperature (Thermo Fisher Scientific, #15250061).
6. Sterile disposable PETG flask: 125 mL with a plain bottom with vented closure (Thermo Fisher Scientific, #4116-0125).
7. Serological pipets: 5 mL sterile disposable polystyrene pipets (Thermo Fisher Scientific, #170373N).
8. Serological pipets: 50 mL sterile disposable polystyrene pipets (Thermo Fisher Scientific, #170358T).
9. 15 mL sterile centrifuge tube (Fisher Scientific, #22-010-075).

2.3.1 Insect Cell Growth and Maintenance

2.3.2 Insect Cell Transfection

10. 6-well plate: Nunc™ cell-culture treated multidishes (Thermo Fisher Scientific, #140675).
11. FuGENE HD transfection reagent: non-liposomal formulation designed to transfect DNA into a wide variety of cell lines, stored at 4 °C (Promega, #E2311).

2.3.3 Baculovirus Generation and Protein Expression

1. 15 mL sterile centrifuge tube (Fisher Scientific, #22-010-075).
2. Sterile disposable PETG flask: 250 mL flask with plain bottom and vented closure (Thermo Fisher Scientific, #4112-0250).
3. Sterile disposable PETG flask: 1000 mL flask with plain bottom and vented closure (Thermo Fisher Scientific, #4115-1000).
4. 50 mL sterile centrifuge tube (Fisher Scientific, #14-432-22).
5. Nalgene™ 500 mL super-speed centrifuge bottles: 500 mL centrifuge bottle with sealing closure (Millipore Sigma, #Z353744).
6. Phosphate-buffered saline (PBS), pH 7.2, stored at 4 °C (Thermo Fisher Scientific, #20012027).

2.4 Protein Purification

2.4.1 Protein Binding Via FLAG Tag

1. Anti-flag M2 affinity gel: monoclonal anti-flag M2 antibody produced in mouse and stored at −20 °C (Millipore Sigma, #A2220).
2. HEPES (N-2-hydroxyethylpiperazine-N′-(2-ehtanesulfonic acid): a zwitterionic organic chemical buffering agent commonly used in cell culture media (Millipore Sigma, #H3375).
3. DTT (dithiothreitol): quantitatively reduces disulfide bonds and maintains monothiols in a reduced state, dissolved in ddH_2O to a final concentration of 1 M, aliquot and stored at −20 °C (Millipore Sigma, #3483-12-3).
4. ATP.Mg (Adenosine 5′-triphosphate magnesium salt): dissolve ATP.Mg in ddH_2O to a final concentration of 100 mM and aliquot and store at −20 °C (Sigma-Aldrich, #A9187).
5. NaCl (sodium chloride): dissolve NaCl in distilled water to a final concentration of 2.5 M and then store at room temperature (Millipore Sigma, #S9888).
6. Pluronic F-127: dissolve powder in distilled water to a final concentration of 10% (w/v) and store at 4 °C (Millipore Sigma, #2443).
7. Beckman Optima TLX Ultracentrifuge (Beckman Coulter).
8. Beckman TLA-110 rotor with fixed angle (Beckman Coulter, #366735).
9. TLA-110 tube: 3.2 mL, 13 × 56 mm polycarbonate tube (Beckman Coulter, #362305).
10. Econo-Pac Chromatography column: used for gravity-flow chromatography, 1.5 × 12 cm polypropylene columns (BIO-RAD, #7321010).
11. Serological pipets: disposable 5 mL sterile polystyrene pipets (Thermo Fisher Scientific, #170373N).
12. cOmplete mini: EDTA-free protease inhibitor cocktail tablets (Roche, #11836170001).
13. PMSF (phenylmethylsulfonyl fluoride) inhibits serine proteases: dissolve in isopropanol to a final concentration of 100 mM, aliquot and store at −20 °C (Millipore Sigma, #10837091001).
14. Lysis Buffer: 50 mM HEPES, 100 mM NaCl, 2 mM $Mg(Ac)_2$, 1 mM EGTA, 0.2% pluronic F-127 (w/v), 10% glycerol (v/v), store at 4 °C.
15. Dounce homogenizer: 40 mL (Fisher Scientific, #K885300-0040).
16. A Benchmarker™ rocker.

2.4.2 Protein Elution

1. Elution buffer: 50 mM Tris–HCl (pH 7.4), 150 mM KAc, 2 mM $Mg(Ac)_2$, 1 mM EGTA, 10% glycerol (v/v), store at 4 °C.
2. Econo-Pac chromatography columns for gravity flow chromatography: 14 cm high polypropylene columns with a 20 mL bed volume (BIO-RAD, #7321010EDU).
3. 2 mL sterile low retention microcentrifuge tubes (Thermo Fisher Scientific, #3453).
4. FLAG peptide: lyophilized power, used for the competitive elution of FLAG fusion proteins from the Anti-FLAG M2 affinity gel. Dissolve in PBS (pH 7.2) to a final concentration of 5 mg/mL and aliquot and store at −20 °C (Millipore Sigma, #F3290).
5. Amicon™ Ultra-15 Centrifugal Filter Units (50 kDa, 15 mL sample volume): regenerated cellulose membranes that are ideal for protein and nucleic acid purification, concentration, and desalting (Millipore Sigma, #UFC905008).
6. SDS-PAGE gel: 4 to 12%, Bis-Tris, stored at 4 °C (Thermo Fisher Scientific, #WG1403BOX).
7. Sample loading buffer: Pierce™ lane marker reducing sample buffer, stored at −20 °C (Thermo Fisher Scientific, #39000).
8. Acrylamide gel electrophoresis system.
9. SDS-PAGE running buffer: NuPAGE™ Tris–acetate SDS running buffer, stored at room temperature (Thermo Fisher Scientific, #LA0041).
10. InstantBlue Coomassie Protein Stain (or similar) stored at 4 °C.
11. Gel imaging system.

2.5 Single-Molecule Assay

2.5.1 Microtubule Polymerization

1. PIPES solution: dissolve piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) in distilled water to a final concentration of 500 mM, adjust the pH to 6.8 with NaOH pellets, and store at 4 °C (Millipore Sigma, P6757).
2. BRB80 with 10% glycerol (BRB80G10): 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EDTA, 10% glycerol (v/v), pH 6.8, stored at 4 °C.
3. BRB80 with 60% glycerol (BRB80G60): 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EDTA, 60% glycerol (v/v), pH 6.8, stored at 4 °C.
4. Tubulin (Rhodamine labeled): dissolve porcine brain tubulin modified to contain covalently linked rhodamine at random surface lysine in 10 μL BRBG10 and aliquot to 2 × 5 μL (final 1 mg/mL) and store at −20 °C (Cytoskeleton, #TL590M).

5. Tubulin (Biotin tagged): dissolve porcine brain tubulin modified to contain covalently linked, long-chain biotin derivative in 20 μL BRBG10 and aliquot to 4 × 5 μL (final 1 mg/mL) and store at −20 °C (Cytoskeleton, #T333P).
6. Tubulin (>99% Pure): 1 mg of white lyophilized powder. Dissolve in 100 μL BRB80G10 and aliquot to 20 × 5 μL(final 10 mg/mL), store at −20 °C (final 10 mg/mL) (Cytoskeleton, #T240).
7. GTP solution (100 mM): dissolve in BRB80G10 to a final concentration of 10 mM, aliquot and store at −20 °C (Thermo Fisher Scientific, #R0461).
8. Paclitaxel (taxol): dissolve in DMSO to a final concentration of 10 mM, aliquot and store at −20 °C (Sigma Aldrich, #T7402).
9. TLA-100 fixed angle rotor (Beckman Coulter, #343840).
10. TLA-100 rotor tube: polycarbonate tube, 7 × 20 mm (Beckman Coulter, #343775).

2.5.2 Microtubule Slide Preparation

1. Biotinylated BSA (bovine serum albumin) solution: dissolve biotinylated BSA in BRBG10 to a final concentration of 5 mg/mL, aliquot and store at −20 °C (Thermo Fisher Scientific, #29130).
2. BSA: dissolve BSA in BRBG10 to a final concentration of 50 mg/mL, aliquot and store at −20 °C (Millipore Sigma, #A9418).
3. Streptavidin: dissolve powder in BRB80G10 to a final concentration of 1 mg/mL, aliquot and store at −20 °C (Thermo Fisher Scientific, #21122).
4. Pluronic F-127: dissolve powder in ddH2O to a final concentration of 10% (w/v) and store at 4 °C (Millipore Sigma, #P2443).
5. A humidity box.
6. BRB80 with 1% Pluronic F-127: 80 mM PIPES, 2 mM $MgCl_2$, 1 mM EGTA, 1% Pluronic F-127, pH 6.8.
7. Wash buffer (WB): add 8 μL of 50 mg/mL BSA (2 mg/mL final) to 192 μL of BRB80 with 1% Pluronic F-127, aliquot to 200 μL/vail and store at −20 °C.
8. Blocking buffer (BB): add 60 μL of 50 mg/mL BSA (1.5 mg/mL final) and 4 μL of 10 mM taxol (10 μM final) to 1.94 mL of BRB80 with 1% Pluronic F-127, aliquot to 200 μL/vail, and store at −20 °C.

2.5.3 Single-Molecule Fluorescent Imaging

1. TCEP: Tris(2-carboxyethyl)phosphine hydrochloride (a strong reducing agent), dissolve in distilled H_2O to a final

concentration of 100 mM, aliquot and store at −20 °C (Millipore Sigma, #C4706).

2. KAc: potassium acetate, dissolve in distilled water to a final concentration of 2.5 M, aliquot and store at 4 °C (Millipore Sigma, #P1190).
3. Glucose: dissolve in distilled water to a final concentration of 1 M, aliquot and store at −20 °C (Millipore Sigma, #G8270).
4. Biotin: dissolve powder in ddH_2O to a final concentration of 50 mM and store at −20 °C.
5. Glucose oxidase: dissolve lyophilized powder in 50 mM HEPES with 50% glycerol to a final concentration of 10 μM. Store at −20 °C and use within 1 month (Millipore Sigma, #C40).
6. Catalase: dissolve lyophilized powder (from bovine liver) in 50 mM HEPES with 50% glycerol to a final concentration of 5 μM. Store at −20 °C and use within 1 month (Millipore Sigma, #G2133).
7. Gloxy: prepare by mixing glucose oxidase and catalase in a 1:1 ratio (v/v). Store at −20 °C and use within 1 month.
8. HME30 buffer (HME30): 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, pH 7.2.
9. HME30 buffer with 50 mM KAc (HME30K50): 30 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA, 50 mM KAc, pH 7.2.
10. Motility buffer (MB): add 60 μL of 50 mg/mL BSA (1.5 mg/mL final), 10 μL of 100 mM TCEP, 40 μL of 1 M glucose and 0.4 μL of 10 mM taxol (10 μM final) to 1.92 mL of HME30K50, aliquot to 200 μL/vail and store at −20 °C.
11. ATP (*see* Subheading 2.4.1).
12. A total internal reflection fluorescence (TIRF) microscope.

2.5.4 Optical Trapping Assay

1. AntiGFP antibody-coated polystyrene beads of 1 μm diameter [24].
2. Motility buffer (MB): add 60 μL of 50 mg/mL BSA (1.5 mg/mL final), 10 μL of 100 mM TCEP, 40 μL of 1 M glucose, and 4 μL of 10 mM taxol (10 μM final) to 1.92 mL of HME30K50, aliquot to 200 μL/vail, and store at −20 °C.
3. An optical tweezers setup.

3 Methods

3.1 Bacmid Generation

This protocol describes the preparation of a bacmid selection plate for blue-white screening and the generation of a bacmid by the transformation of competent DH10Bac cells. The blue-white

screening relies on the activity of β-galactosidase, which cleaves lactose into glucose and galactose.

3.1.1 Preparation of the Bacmid Selection Plate

1. Measure 3.5 g LB-agar powder and transfer it into a 250 mL autoclaving bottle.
2. Add 100 mL distilled water to the bottle and swirl to form a medium/agar colloid.
3. Place the gel mix in the autoclave and heat the sample to 121 °C under 20 psi for 30 min.
4. Prepare a water bath set at 60 °C with sufficient water to submerge 75% of the bottle containing the gel mixture (*see* **Note 1**).
5. Carefully transfer the autoclave bottle into the water bath and leave the molten gel-mix for at least 5 min.
6. Light the flame at the plate pouring station. Add 70 μL of gentamycin (7 μg/mL final), 1 mL tetracycline (10 μg/mL final), 167 μL of kanamycin (50 μg/mL final), 80 μL of IPTG (40 μg/mL final), and 500 μL of bluo-gal (100 μg/mL final) to the gel mixture (*see* **Notes 2** and **3**).
7. Pour directly from the bottle to plates.
8. Stack plates together. Cover the plate stack with aluminum foil to protect from light and leave plates out on the bench to solidify.
9. Store plates at 4 °C in dark until use (*see* **Note 4**).

3.1.2 Recombinant Bacmid Generation

1. Place one tube of frozen DH10Bac cells (100 μL) on ice and let sit for 5 min.
2. Add 50 ng plasmid DNA of interest to DH10Bac cells and gently tap the tube to mix (*see* **Note 5**).
3. Incubate cells on ice for 30 min.
4. After incubation, heat-shock cells in a 42 °C water bath for 45 s (*see* **Note 6**).
5. Place the tube immediately back on ice and incubate for 2 min.
6. Add 0.9 mL room temperature "Super Optimal broth with Catabolite repression" (S.O.C.) medium.
7. Shake the tube at 225 rpm for 4 h at 37 °C to recover.
8. After shaking, transfer 200 μL cell solution to a new tube and dilute with 800 μL S.O.C. medium that is kept at room temperature. Mix well by pipetting and spread 100 μL solution onto a blue-white selection plate.
9. Place the plate in a 37 °C incubator for 24 h (*see* **Note 7**).

3.2 Bacmid Purification

This protocol describes the growth and purification of high purity and highly concentrated bacmids for subsequent insect cell transfection. Bacmid DNA is extracted from overnight culture using an isopropanol precipitation method with Qiagen buffer [25].

3.2.1 Bacmid Culture Preparation

1. Light the flame of a Bunsen Burner at the experimentation site. Add 7 μL of gentamycin (7 μg/mL final), 100 μL tetracycline (10 μg/mL final), and 16.7 μL of kanamycin (50 μg/mL final) to 10 mL of LB broth medium and mix well.
2. Transfer 3 mL of above LB broth selection medium to a 14 mL round-bottom test tube.
3. Use a sterilized wood applicator stick to pick a single, isolated white colony from the bacmid selection plate and drop into the test tube containing selection medium (*see* **Note 8**).
4. Incubate cells overnight at 37 °C with shaking (*see* **Note 9**).

3.2.2 Bacmid Culture Purification

1. Transfer the overnight culture into 2 mL microcentrifuge tubes and spin at 4000 rpm for 5 min using a microcentrifuge.
2. Remove supernatant and resuspend cell pellet with 0.3 mL P1 solution from QIAGen spin miniprep kit.
3. Add 0.3 mL P2 solution into the tube and mix well by gently pipetting. Incubate the mixture at room temperature for 5 min.
4. After the incubation, add 0.4 mL P3 solution to the tube and mix gently by inverting the tube slowly. Incubate the mixture on ice for 6 min (*see* **Note 10**).
5. Centrifuge the solution at 13,000 rpm for 10 min.
6. Carefully transfer the supernatant to a new 2 mL microcentrifuge tube and spin again at 13,000 rpm for 2 min (*see* **Note 11**).
7. Transfer supernatant to a 2 mL microcentrifuge containing 0.8 mL isopropanol and invert the tube to mix well.
8. Place the tube on ice and incubate for 10 min.
9. After incubation, centrifuge at 13,000 rpm for 10 min.
10. Carefully remove the supernatant and add 0.5 mL 70% ethanol to the tube. Invert the tube several times to wash the pellet.
11. Centrifuge again at 13,000 rpm for 5 min.
12. Repeat ethanol wash step (**steps 10** and **11**) for two more times and carefully remove the supernatant.
13. Light the flame of a Bunsen Burner at the experimentation site. Open the tube cap and airdry the DNA pellet for 1 min.
14. Dissolve DNA pellet in 50 μL sterilized QIAgene EB buffer by gently tapping the tube (*see* **Note 12**).
15. Store the bacmid at 4 °C until use (*see* **Note 13**).

3.3 Insect Cell Transfection, Baculovirus Generation, and Protein Expression

This protocol describes how to thaw a frozen stock of sf9 insect cells and how to maintain a sf9 insect cell culture, how to transfect insect cells and how to generate and amplify baculoviruses, and how to express proteins in insect cells [20, 26].

3.3.1 Insect Cell Growth and Maintenance

1. Warm Sf-900 Serum Free Medium (SFM) to room temperature and decontaminate the biosafety hood with UV light.
2. Take out one vial of frozen Sf9 insect cells from liquid nitrogen and quickly thaw the cells in a 37 °C water bath by submerging 2/3 of the vial under the water until only a small ice crystal remains.
3. Thoroughly decontaminate the cryovial with 70% ethanol and transfer it to the biosafety hood.
4. Carefully transfer cells with a sterile 2 mL serological pipet to a 15 mL sterile centrifuge tube.
5. Drop-wise add 3 mL pre-warmed medium to resuspend the cells and gently swirl the tube while adding the medium.
6. Transfer 0.2 mL cell suspension to a new tube and perform a cell counting using trypan blue to determine the cell density, viability, and health.
7. Transfer cell suspension into a 125 mL sterile flask. According to the calculated cell density, dilute the cells with medium to a final density of 0.8×10^6 viable cells/mL.
8. Incubate cells in a non-humidity incubator for 4 days at 27 °C with shaking at 125 rpm.
9. After 4 days, determine the cell density and viability again. If the cell density is lower than 2×10^6 viable cells/mL, wait for one more day for the cell to grow and check the cell density again.
10. If the cell density reaches $>2 \times 10^6$ viable cells/mL, passage cells by diluting with Sf9 SFM to a final density of 0.6×10^6 cells/mL.
11. Passage cells every 3–4 days when the cell density reaches 2×10^6 cells/mL. Do not overgrow cells to a density higher than 4×10^6 cells/mL.

3.3.2 Insect Cell Transfection

1. In a biosafety hood, transfer 2 × 2 mL of Sf9 cells at the density of 0.5×10^6 cells/mL to a 6-well plate and leave the plate in room temperature to allow cells to attach (*see* **Note 14**).
2. Add 198 μL of SF900 SFM and 2 μg of bacmid DNA into a Falcon round-bottom tube and gently mix by tapping.

3. Add 6 μL of FuGENE HD transfection reagent directly to the medium/DNA mixture and incubate at room temperature for 15 min (*see* **Note 15**).
4. After incubation, add FuGENE/bacmid mixture to cells dropwise by distributing around the well. Supply equal amount of SF900™II SFM to the well of control group. Then shake gently in the X and Y direction (*see* **Note 16**).
5. Place the 6-well plate in a clean, sterilized box with damp tissue around the plate to keep it moist.
6. Thoroughly disinfect the box with 70% ethanol and incubate the cells at 27 °C for 4 days.

3.3.3 Baculovirus Generation and Protein Expression

1. After 4 days incubation, transfer the supernatant containing virus from the well to a sterile 15 mL centrifuge tube. Label the tube as P1 virus and store it at 4 °C in dark (*see* **Note 17**).
2. Transfer 50 mL sf9 cells at 1.5×10^6 cells/mL to a 250 mL sterile disposable flask and add 0.5 mL P1 viral supernatant to the cells (*see* **Note 18**).
3. Incubate the cells at 27 °C with shaking for 3 days to produce P2 virus.
4. After incubation, transfer the cell culture to a 50 mL centrifuge tube and spin at 4 °C, 2500 rpm for 10 min using a centrifuge.
5. Carefully transfer the supernatant into a new 50 mL tube and label as P2 virus. Store the tube at 4 °C in dark and use within 1 month (*see* **Note 19**).
6. Transfer 350 mL sf9 cells at 2×10^6 cells/mL to a 1 L sterile disposable flask and add 3.5 mL P2 virus to the cells (*see* **Note 20**).
7. Incubate at 27 °C with shaking for 60–72 h (*see* **Note 21**).
8. Transfer the cell culture to a 500 mL centrifuge bottle and spin at 2000 g for 15 min.
9. Discard the supernatant and resuspend the cell pellet with 40 mL cold PBS.
10. Transfer the solution to a 50 mL centrifuge tube and spin again at 2000 g for 10 min.
11. Discard the supernatant and flash freeze the cell pellet with liquid nitrogen. Store the cell pellet at −80 °C until use (*see* **Note 22**).

3.4 Protein Purification

The kinesin motor protein KIF5C is purified via a FLAG-tag at the C-terminus. Subheading 3.4.1 describes the binding of the motor protein to the anti-FLAG agarose affinity gel, and Subheading 3.4.2 describes the elution of the protein from the gel by incubating with the FLAG peptide.

3.4.1 Protein Binding Via FLAG Tag

1. All purification steps are performed in a cold room at 4 °C.
2. Cool down a Dounce homogenizer and put TLA-110 spin tubes on ice.
3. Place the tube containing the frozen cell pellet in a beaker with water at room temperature to slowly thaw the pellet. Once the sides of the ice are thawed, transfer the tube immediately onto ice.
4. In the meantime, prepare 50 mL lysis buffer and supply with ATP (final 0.1 mM) and DTT (final 1 mM). Chill the solution on ice.
5. Dissolve 1 tablet of protease inhibitor into the 50 mL above buffer to obtain the final lysis buffer (LB).
6. Add 20 mL LB to the tube with cell pellet and mix by gently inverting.
7. Once most of cells are thawed, supply the solution with PMSF (final 800 μM) and mix gently by inverting.
8. Transfer the cell lysate to a Dounce homogenizer and dounce with 20 strokes (*see* **Note 23**).
9. Transfer the cell lysate to TLA-110 tubes and clear the lysate by centrifugation at 80,000 rpm (average 260,000 × g), 4 °C for 10 min in a Beckman tabletop ultracentrifuge.
10. In the meantime, prepare the anti-FLAG M2 beads for purification: transfer 500 μL anti-FlAG M2 beads to a 15 mL centrifuge tube containing 4.5 mL LB. Gently invert the tube to mix. Centrifuge the tube at 500 × g, 4 °C for 2 min. Carefully remove the supernatant without disturbing the beads on the bottom. Repeat these wash steps for 2 more times (*see* **Note 24**).
11. After centrifugation (**steps 8** and **9**), collect supernatant and transfer to a 50 mL centrifuge tube. Transfer washed anti-FLAG beads to the same tube and seal the cap with parafilm (*see* **Note 25**).
12. Place the tube on a rocker and incubate at 4°C with rotation for 2 h. Store the rest of LB (~20 mL) at 4 °C.

3.4.2 Protein Elution

1. Prepare 20 mL elution buffer and supply with ATP (final 0.1 mM) and DTT (final 1 mM) to obtain the final elution buffer (EB). Chill the buffer on ice.
2. After incubation, transfer the protein/beads solution to a 20 mL gravity column and make sure the valve is closed. Wait 5 min for the resin to settle (*see* **Note 26**).
3. Open the valve to allow dripping. Once the solution level is close to the top of the resin, close the valve.

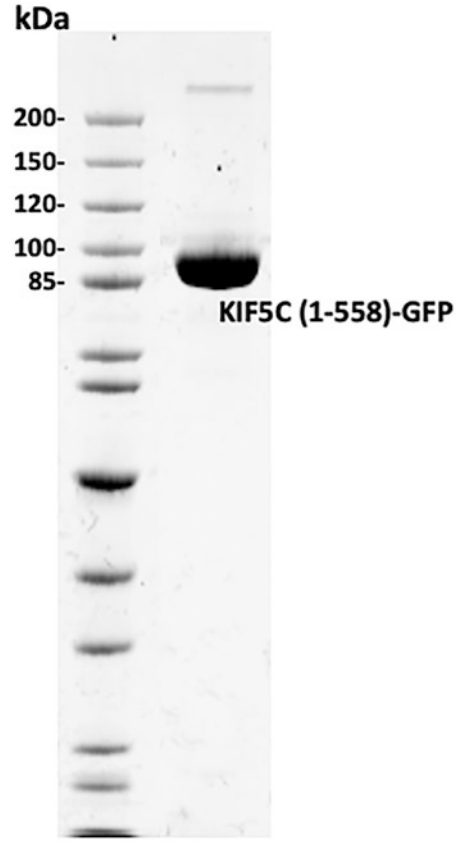

Fig. 2 FLAG tag-based purification of KIF5C(1-558)-GFP. 4–12% Bis-Tris SDS-PAGE stained with InstantBlue™

4. Add 5 mL LB and start dripping. Repeat this step two more times (*see* **Note 7**).
5. After the LB rinse, add 5 mL EB and start dripping. Repeat this step two more times. Close the valve after the EB rinse.
6. Cut the tip of a 1 mL pipet tip and use it to carefully transfer the beads to a 2 mL low-retention microcentrifuge tube (*see* **Note 28**).
7. Supply the resin with EB to reach 1.5 mL. Add 45 μL, 5 mg/mL FLAG peptide and incubate on ice for 15 min.
8. Spin at 500 × g for 1 min and collect the supernatant to a new low-retention tube.
9. Repeat **steps 7** and **8** for three more times and collect all supernatant together (*see* **Note 29**).
10. To remove FLAG peptide and concentrate the protein, transfer supernatant to a 15 mL Amicon centrifuge filter and centrifuge at 6000 × g, 4 °C for 3 min.

11. Resuspend the solution in the filter by careful pipetting and repeat the centrifugation step (*see* **Note 30**).
12. Stop the centrifugation once the desired volume is reached.
13. Aliquot the protein solution into 50 μL/vial and 2 μL/vial. Flash freeze in liquid nitrogen. Store the proteins at −80 °C.
14. Run SDS-PAGE gel to confirm the successful protein purification. Figure 2 shows the SDS-PAGE gel of KIF5C(1-558)-GFP purified from insect cells.

3.5 Single-Molecule Assay

TIRF microscopy and optical tweezers are frequently used to study the properties of microtubule-based motor proteins in vitro, including velocity, processivity (the ability to take multiple steps before dissociating), and force generation. In this protocol, biotinylated microtubules are attached to a coverslip through streptavidin and BSA-biotin that is nonspecifically bound to the coverslip surface. Fluorescently tagged motor proteins and ATP are then introduced into the chamber, and the movement of motors along microtubules is visualized using TIRF microscopy. To measure the force generation of the purified motors, GFP-tagged motor proteins are bound to anti-GFP antibody-coated polystyrene trapping beads and flown into the slide chamber. Using a custom-built or commercial optical tweezers setup, detachment and stall forces, step sizes, and force-velocity relationships can be measured.

3.5.1 Microtubule Polymerization

1. Mix one aliquot of 5 μL, 10 mg/mL tubulin, one aliquot of 5 μL, 1 mg/mL rhodamine labeled tubulin and one aliquot of 5 μL, 1 mg/mL biotin-tagged tubulin by gently pipetting.
2. Add 1.5 μL, 10 mM GTP (final 1 mM) to the tubulin mixture and mix by gently pipetting.
3. Incubate the mixture at 37 °C for 20 min.
4. Add 1.5 μL of 0.2 mM taxol (in DMSO) to the tubulin mixture and incubate at 37 °C for 10 min.
5. Add 0.1 μL of 10 mM taxol (in DMSO) to 100 μL of BRB80G60 (cushion) and transfer 60 μL of the cushion to a TLA100.1 tube.
6. Add 0.2 μL of 10 mM taxol (in DMSO) and 1 μL of 0.1 M DTT to 100 μL of BRB80G10 (BRB).
7. After the incubation, carefully layer the tubulin mixture on top of the glycerol cushion in the TLA100.1 tube and spin at 80,000 rpm (260,000 × g) for 10 min.
8. Remove supernatant and wash the pellet by carefully pipetting 20 μL BRB buffer on the pellet, then remove the solution immediately. Repeat the wash step twice.

9. Resuspend the pellet with 10 μL of BRB by gentle pipetting (final concentration of 6 mg/mL). Store the microtubule solution at room temperature in dark.

3.5.2 Microtubule Slide Preparation

The coverslips are cleaned and the flow chambers are assembled as described previously [24]. This protocol focuses on the method of immobilizing microtubules on the coverslip surface.

1. Clean coverslip and assemble microscope slide chamber as described previously [24].
2. Flow 12 μL of 5 mg/mL BSA-biotin into the microtubule slide chamber and incubate in a humidity box at room temperature for 5 min.
3. Wash chamber three times with 20 μL WB, then incubate the flow chamber in a humidity box at room temperature for 30 min (*see* **Note 31**).
4. Wash the chamber with 20 μL BB twice. Completely remove the solution from the flow chamber using vacuum. Flow in 12 μL 1 mg/mL streptavidin solution and incubate at room temperature in the humidity box for 5 min.
5. Dilute fluorescently labeled, biotinylated microtubules with BB to a final concentration of 0.5 mg/mL, then add 1 μL of diluted microtubules to 20 μL BB and mix well by gently pipetting.
6. Completely remove the streptavidin solution from the flow chamber using vacuum and wash twice with 20 μL BB.
7. Remove BB from the chamber with vacuum and immediately flow in 20 μL BB containing microtubules, then carefully wash the chamber twice with 20 μL BB.

3.5.3 Single-Molecule Fluorescent Imaging

1. Transfer one aliquot of the purified motor proteins to ice and let the solution in the aliquot thaw (*see* **Note 32**).
2. In the meantime, exchange the buffer in the prepared microtubule slide chamber with 20 μL MB.
3. Mix 0.8 μL of gloxy (final 2 μM), 0.8 μL of biotin (final 1 mM) and 0.8 μL of ATP (final 2 mM) to 38 μL of MB and mix well by pipetting, then add 1 μL of an appropriately diluted motor stock and mix well.
4. Flow 2 × 20 μL of the mixture into the chamber.
5. Seal the chamber using vacuum grease.
6. Mount the slide on a TIRF microscope and adjust the laser power to obtain a good signal-to-noise ratio without causing a significant photo bleaching of the fluorophores. Acquisition times of 100–200 ms per frame (or faster) are preferred as KIF5C is relatively fast.

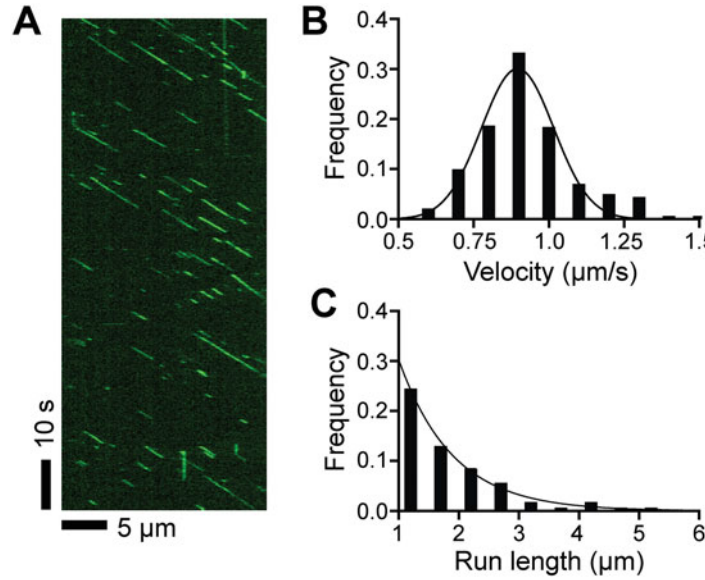

Fig. 3 (**a**) A kymograph example showing processive motion of KIF5C (1-558)-GFP. (**b**) Velocity histograms of KIF5C (1-558) (0.89 ± 0.04 μm/s, mean ± SEM from Gaussian fit; $n = 347$). (**c**) Statistical analysis of run length of KIF5C (0.89 μm, $n = 347$)

7. Analyze the acquired images using Image J and calculate the motor's velocity and processivity with Prism. Figure 3a depicts an example kymograph of KIF5C molecules moving along microtubules. Figure 3b, c shows the calculated velocity and run length of KIF5C, respectively.

3.5.4 Optical Trapping Assay

This protocol describes how to perform force measurement on microtubule-associated motor proteins using optical tweezers. We start with binding of the GFP-tagged motor proteins to anti-GFP antibody-coated polystyrene trapping beads. The beads are then introduced into a slide chamber with surface-attached microtubules (preparation described in Subheading 3.5.2). After the slide chamber is mounted to the microscope (our optical tweezers setup is combined with TIRF microscopy to visualize the fluorescently-labeled microtubes), beads are captured with the optical trap and placed above a surface-bound microtubule. If a motor is bound to the trapped bead, force generation along the microtubule can be measured.

1. Prepare a microtubule slide chamber as described in Subheading 3.5.2.
2. Transfer one aliquot of purified motor proteins to ice and wait until the aliquot with the protein solution is thawed (*see* **Note 32**).

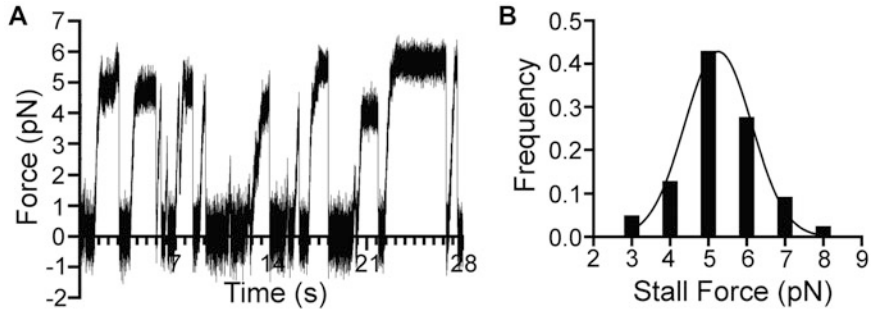

Fig. 4 (**a**) Representative force versus time records of bead movement driven by single molecules of kinesin-1 KIF5C(1-558). (**b**) Stall force histograms of KIF5C(1-558) (5.25 ± 0.07 pN, mean ± SEM from Gaussian fit; stall plateaus ≥200 ms; $n = 163$)

3. Add 0.5 μL of anti-GFP antibody-coated polystyrene beads to 19 μL MB and mix well. Then 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, exchange the buffer in the prepared microtubule slide chamber with 20 μL MB.
5. Mix 0.8 μL of gloxy (final 2 μM), 0.8 μL of biotin (final 1 mM) and 0.8 μL of ATP (final 2 mM) to 20 μL of MB and mix well by pipetting. Then add this buffer to the beads/motor mixture and mix by gently pipetting.
6. Flow 2 × 20 μL of mixture into the chamber.
7. Seal the chamber using vacuum grease.
8. Mount the slide on the microscope and adjust and find a straight microtubule using the TIRF imaging mode.
9. Trap a bead and place the bead over a surface-bound microtubule and wait for an appropriate time to determine if movement and force generation can be observed (*see* **Note 33**).
10. Record the data (using custom or commercial software) and then analyze the acquired data using a custom-written MATLAB program. Figure 4a shows representative force versus time records of bead movement driven by single kinesin-1 KIF5C(1–558) molecule. Figure 4b shows the histograms of stall forces (5.25 ± 0.07 pN, mean ± SEM from Gaussian fit; stall plateaus ≥200 ms; $n = 163$).

4 Notes

1. Antibiotics become unstable at high temperature. Cool the molten gel mixture in the water bath before adding the antibiotics.

2. The bluo-gal used in the selection plates is stable when stored in powder form at −20 °C but is unstable when stored at 4 °C in the agar plates. It is essential to prepare fresh plates to ensure the best blue-white screening efficiency.
3. Swirl the agar bottle to ensure even distribution of antibiotics throughout the gel.
4. Bluo-gal is unstable under light. Cover plates with aluminum foil to protect from light.
5. The pFastBac plasmid used in this study contains the DNA for the expression of a tail-truncated KIF5C(1-558) construct with a C-terminal FLAG-tag for purification and a green fluorescence protein for single-molecule imaging and force measurements [27].
6. Do not shake or vortex cells.
7. Shorter incubation times can result in difficulties interpreting the blue-white selection.
8. True white colonies tend to be larger in size. Select the most isolated colonies to avoid cross-contamination.
9. Optional: before starting the miniprep for bacmid purification, streak from the culture a wedge on a bacmid selection plate and incubate the plate at 37 °C overnight. Proceed with the purification if the culture produces white colonies only.
10. A thick and white pellet forms after gently mixing by slowly inverting the tube.
11. Spin again for 2 min to remove any residual chromatin flakes.
12. Place the tube on ice to assist the dissolving of the pellet.
13. Do not freeze bacmids. Bacmids are usually stable for 1 month when stored at 4 °C. Prepare fresh bacmids for better transfection efficiency.
14. Transfer 2 × 2 mL of Sf9 cells into two separate wells in a 6-well plate. One well is used for the experiment and the second well as a control.
15. Do not allow the undiluted transfection reagent to contact the side of the tube.
16. Do not circular swirl the plate as this will distribute cells to the edge.
17. Examine the transfected Sf9 cells in the 6-well plate under a microscope. Compared to the control, enlargement of the nucleus and growth cessation indicates successful transfection. If the protein to be expressed has a fluorescent protein, examine the cells under a fluorescent microscope and proceed if a moderate amount of fluorescence is observed.

18. Count the stock cells and ensure cell viability is above 95% for subsequent experiments.
19. The P2 virus stock should be used within 1 month. For larger constructs such as human dynein, the P2 virus should be used within 2 weeks.
20. Use a higher volume of insect cells for protein expression of larger constructs. Add the P2 virus to cells at a ratio of 1: 100 v/v.
21. For sensitive proteins, harvest earlier to avoid lysed cells releasing proteases, which will lead to protein degradation.
22. Make sure the tube cap is not tightly closed. Place the tube in liquid nitrogen until the vigorous boiling stabilizes.
23. Gently dounce to avoid bubble formation, as some proteins tend to aggrege when in air contact.
24. Use 100 μL anti-FLAG M2 affinity beads per 100 mL cell lysate and adjust the used volume accordingly.
25. For proteins that tend to aggregate when in air contact, select a centrifuge tube of appropriate volume and fill the tube to the very top with LB. This is of particular importance to avoid aggregation of large molecular weight proteins, such us human dynein.
26. Rinse the gravity column with 2 mL LB before transferring the protein/beads solution to minimize the non-specific binding of protein to the column walls. Avoid creation of bubbles when transfer the solution.
27. For protein that is sticky, rinse with a higher volume of buffer to remove impurities.
28. After the transfer of the protein/beads solution to the low-retention microcentrifuge tube, add stepwise 0.2 mL of EB to the column to transfer the remaining beads if necessary.
29. If the protein to be purified contains a fluorescent tag, successful elution could be visualized by the color change of the resin from color to colorless.
30. Rinse the filter with EB before centrifugation to minimize protein adhesion to the filter membrane. It is essential to resuspend the solution after each centrifugation to avoid protein sticking to the filter membrane and aggregation due to high concentration close to the membrane.
31. The BSA and Pluronic F-127 are both good blocking reagents for coverslips to prevent molecular motors or beads from non-specifically binding to the coverslip surface.
32. Do not warm the protein in your hands as this could cause the protein to aggregate.

33. 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 microtubules [28].

References

1. Hoyt MA, Hyman AA, Bahler M (1997) Motor proteins of the eukaryotic cytoskeleton. Proc Natl Acad Sci U S A 94(24):12747–12748. https://doi.org/10.1073/pnas.94.24.12747
2. Xiao Q, Hu X, Wei Z, Tam KY (2016) Cytoskeleton molecular motors: structures and their functions in neuron. Int J Biol Sci 12(9):1083–1092. https://doi.org/10.7150/ijbs.15633
3. Hirokawa N, Noda Y, Okada Y (1998) Kinesin and dynein superfamily proteins in organelle transport and cell division. Curr Opin Cell Biol 10(1):60–73
4. Abraham Z, Hawley E, Hayosh D, Webster-Wood VA, Akkus O (2018) Kinesin and dynein mechanics: measurement methods and research applications. J Biomech Eng 140(2). https://doi.org/10.1115/1.4037886
5. Hirokawa N (1998) Kinesin and dynein superfamily proteins and the mechanism of organelle transport. Science 279:519–526
6. Roberts AJ, Kon T, Knight PJ, Sutoh K, Burgess SA (2013) Functions and mechanics of dynein motor proteins. Nat Rev Mol Cell Biol 14:713–726
7. Hirokawa N, Noda Y, Tanaka Y, Niwa S (2009) Kinesin superfamily motor proteins and intracellular transport. Nat Rev Mol Cell Biol 10
8. Reck-Peterson SL, Yildiz A, Carter AP, Gennerich A, Zhang N, Vale RD (2006) Single-molecule analysis of dynein processivity and stepping behavior. Cell 126(2):335–348. https://doi.org/10.1016/j.cell.2006.05.046
9. Gennerich A, Carter AP, Reck-Peterson SL, Vale RD (2007) Force-induced bidirectional stepping of cytoplasmic dynein. Cell 131(5):952–965
10. Liu X, Rao L, Gennerich A (2020) The regulatory function of the AAA4 ATPase domain of cytoplasmic dynein. Nat Commun 11:5952. https://doi.org/10.1038/s41467-020-19477-3
11. Kost TA, Condreay JP, Jarvis DL (2005) Baculovirus as versatile vectors for protein expression in insect and mammalian cells. Nat Biotechnol 23(5):567–575. https://doi.org/10.1038/nbt1095
12. Airenne KJ, Peltomaa E, Hytonen VP, Laitinen OH, Yla-Herttuala S (2003) Improved generation of recombinant baculovirus genomes in Escherichia coli. Nucleic Acids Res 31(17):e101. https://doi.org/10.1093/nar/gng102
13. Pijlman GP, van Schijndel JE, Vlak JM (2003) Spontaneous excision of BAC vector sequences from bacmid-derived baculovirus expression vectors upon passage in insect cells. J Gen Virol 84(Pt 10):2669–2678. https://doi.org/10.1099/vir.0.19438-0
14. Zhao Y, Chapman DA, Jones IM (2003) Improving baculovirus recombination. Nucleic Acids Res 31(2):E6–E6. https://doi.org/10.1093/nar/gng006
15. Laitinen OH, Airenne KJ, Hytonen VP, Peltomaa E, Mahonen AJ, Wirth T, Lind MM, Makela KA, Toivanen PI, Schenkwein D, Heikura T, Nordlund HR, Kulomaa MS, Yla-Herttuala S (2005) A multipurpose vector system for the screening of libraries in bacteria, insect and mammalian cells and expression in vivo. Nucleic Acids Res 33(4):e42. https://doi.org/10.1093/nar/gni042
16. Felberbaum RS (2015) The baculovirus expression vector system: a commercial manufacturing platform for viral vaccines and gene therapy vectors. Biotechnol J 10(5):702–714. https://doi.org/10.1002/biot.201400438
17. Shi X, Jarvis DL (2007) Protein N-glycosylation in the baculovirus-insect cell system. Curr Drug Targets 8(10):1116–1125. https://doi.org/10.2174/138945007782151360
18. Harrison RL, Jarvis DL (2006) Protein N-glycosylation in the baculovirus-insect cell expression system and engineering of insect cells to produce "mammalianized" recombinant glycoproteins. Adv Virus Res 68:159–191. https://doi.org/10.1016/S0065-3527(06)68005-6
19. Chen N, Kong X, Zhao S, Xiaofeng W (2020) Post-translational modification of baculovirus-encoded proteins. Virus Res 279:197865. https://doi.org/10.1016/j.virusres.2020.197865

20. Schlager MA, Hoang HT, Urnavicius L, Bullock SL, Carter AP (2014) In vitro reconstitution of a highly processive recombinant human dynein complex. EMBO J 33:1855–1868
21. Korten T, Chaudhuri S, Tavkin E, Braun M, Diez S (2016) Kinesin-1 expressed in insect cells improves microtubule in vitro gliding performance, long-term stability and guiding efficiency in nanostructures. IEEE Trans Nanobioscience 15(1):62–69. https://doi.org/10.1109/TNB.2016.2520832
22. Telley IA, Bieling P, Surrey T (2009) Obstacles on the microtubule reduce the processivity of Kinesin-1 in a minimal in vitro system and in cell extract. Biophys J 96(8):3341–3353. https://doi.org/10.1016/j.bpj.2009.01.015
23. Bird JE, Takagi Y, Billington N, Strub MP, Sellers JR, Friedman TB (2014) Chaperone-enhanced purification of unconventional myosin 15, a molecular motor specialized for stereocilia protein trafficking. Proc Natl Acad Sci U S A 111(34):12390–12395. https://doi.org/10.1073/pnas.1409459111
24. Rao L, Gennerich A (2022) Single-molecule studies on the motion and force generation of the Kinesin-3 motor KIF1A. Methods Mol Biol 2478:585–608. https://doi.org/10.1007/978-1-0716-2229-2_21
25. Zhang K, Foster HE, Rondelet A, Lacey SE, Bahi-Buisson N, Bird AW, Carter AP (2017) Cryo-EM reveals how human cytoplasmic dynein is auto-inhibited and activated. Cell 169(7):1303–1314
26. Htet ZM, Gillies JP, Baker RW, Leschziner AE, DeSantis ME, Reck-Peterson SL (2020) LIS1 promotes the formation of activated cytoplasmic dynein-1 complexes. Nat Cell Biol 22(5):518–525. https://doi.org/10.1038/s41556-020-0506-z
27. Budaitis BG, Badieyan S, Yue Y, Blasius TL, Reinemann DN, Lang MJ, Cianfrocco MA, Verhey KJ (2022) A kinesin-1 variant reveals motor-induced microtubule damage in cells. Curr Biol 32(11):2416–2429 e2416. https://doi.org/10.1016/j.cub.2022.04.020
28. Brenner S, Berger F, Rao L, Nicholas MP, Gennerich A (2020) Force production of human cytoplasmic dynein is limited by its processivity. Sci Adv 6(15):eaaz4295. https://doi.org/10.1126/sciadv.aaz4295