# Chapter 2

# Functional Surface Attachment in a Sandwich Geometry of GFP-Labeled Motor Proteins

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## Abstract

Molecular motors perform work in cells by moving in an ATP-dependent manner along filamentous tracks. In vitro, the mechanical action of such motor proteins can be investigated by attaching the molecules to surfaces in the so-called gliding or bead assays. Surface attachment protocols have to be used that do not interfere with the function of the molecule. Here, we describe a sandwich protocol that preserves functionality. The protocol can be used for a large variety of proteins, in particular kinesin motor proteins that are GFP-tagged.

**Key words:** Motor protein, Surface attachment, Kinesin, Kip3p, Gliding assay, Bead assay

## 1. Introduction

The success of in vitro motility assays depends critically on the attachment of the motor proteins to surfaces, such as plastic or glass beads and glass coverslips or microscope slides. Early success with kinesin-1 was due to its fortuitous attachment to glass surfaces such that some of the motors remained functional and allowed microtubule sliding (1). This probably explains why it was kinesin-1, rather than other kinesins, that was first isolated (2). Later success with protein-coated surfaces (3) and specifically casein-coated surfaces (4) facilitated the development of single-motor assays. However, it turned out that other kinesins were not so easy to reconstitute in vitro, probably because success relies on attachment to surfaces of non-motor domains, which are highly divergent within the kinesin superfamily (5).

The development of new surface-coating protocols tailored for individual motor proteins is very labour-intensive – about

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1 person-year per protocol, in our experience. Thus, the development of a generic protocol is highly advantageous. This chapter describes such a protocol for GFP-tagged proteins (6, 7).

## 2. Materials

### 2.1. Flow Cell

1. Coverslip 22×22 mm and 18×18 mm (Corning, No. 1.5).
2. Parafilm.
3. Hot plate (150°C).
4. Polystyrene (Fluka, Munich, Germany) 1 wt% dissolved in toluene.
5. Spin coater (S.P.S. Vertriebs GmbH, Ingolstadt, Germany).

### 2.2. Gliding and Bead Assay

1. NeutrAvidin (Pierce, Rockford, USA) 1 mg/ml in PBS.
2. Biotinylated monoclonal antiGFP (use EZ-Link NHS-PEO Solid Phase).
3. Biotinylation kit (Perbio GmbH, Bonn, Germany).
4. BRB80: 80 mM PIPES/KOH pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA.
5. Motility buffer: BRB 80 with 1 mM ATP, 0.16 mg/ml casein and varying salt concentration depending on the kind of protein under investigation – use 112 mM KCl for the kinesin-8 Kip3p.
6. Microtubules (8, 9).
7. His6-Kip3p-EGFP (expressed in our lab).
8. Phosphate-buffered saline (PBS), pH 7.4.
9. Pluronic F-127 (Invitrogen).

### 2.3. Bead Assay

1. Polystyrene microspheres: 500 nm in diameter with a concentration of 2.61 wt% (Polyscience, Warrington, USA).
2. Streptavidin-coated microspheres (Sperotech, Lake Forest, USA).
3. Low protein binding tubes (nerbe plus, Winsen/Luhe, Germany).
4. Wash buffer A: PBS with 5 mg/ml biotin-free BSA and 0.1% Tween20.
5. Wash buffer B: PBS with 5 mg/ml biotin-free BSA and 0.1% Tween20 and 0.2 nM biotin.
6. Sonication bath.
7. Thermomixer comfort.
8. Tubulin antibody (SAP.4G5, Sigma Aldrich).

## 3. Methods

### 3.1. Flow Cell

1. Before building the flow cell, the cover glasses are rendered hydrophobic either by silanization (9) or by spin-coating a teflon (10) or polystyrene film (several tens of nanometres thick) onto a glass surface (hold the cover glass with vacuum on the rotating stamp of the spin coater, add 50 µl of dissolved polystyrene or Teflon on the cover glass, rotate cover glass for 10 s at 2,000 rpm and an acceleration of 10,000 rpm, to stabilize the polystyrene coating on the cover glass you can bake them for 30 min to 1 h at 150°C in an oven).
2. The flow cell is constructed out of one 22 × 22 mm and another 18 × 18 mm cover glass separated by two parafilm strips placed next to each other to form channels 100 µm thick, 2 mm wide, and 18 mm long. The parafilm is shortly melted on a hot plate to seal the channel walls.

### 3.2. Gliding Assay

Use filter paper or vacuum to flow given volumes through the channel of the flow cell while always keeping solution in the channel.

#### 3.2.1. Surface Functionalization (8, 9)

1. 20 µl NeutrAvidin (0.1 mg/ml in PBS); incubate 10 min.
2. 20 µl PBS (to wash out unbound NeutrAvidin).
3. 20 µl F-127 (1% in PBS); incubate 10 min.
4. 5 × 20 µl PBS (to wash out unbound F-127).
5. 20 µl biotinylated antiGFP diluted in PBS (the degree of dilution will determine the density of bound GFP-tagged motor proteins in the next steps); incubate 10 min.
6. 3 × 20 µl BRB80 (to wash out unbound antibodies).

#### 3.2.2. Motility Experiment

1. 20 µl motility buffer (equilibration).
2. 20 µl of motility buffer with the GFP-tagged motor protein; incubate 5 min.
3. 3 × 20 µl motility buffer (to wash out unbound motor protein).
4. 20 µl motility buffer with microtubules.
5. The movement of fluorescently labelled microtubules propelled by GFP-labelled proteins can be observed by fluorescence (see Fig. 1) or LED-illuminated video-enhanced differential interference contrast (DIC) microscopy (11).

### 3.3. Bead Assay

*Overview*

1. Prepare or purchase (see Notes 1–5) microspheres functionalized with biotin-binding proteins.
2. Bind biotinylated antibodies via the biotin-tag to the functionalized microspheres.

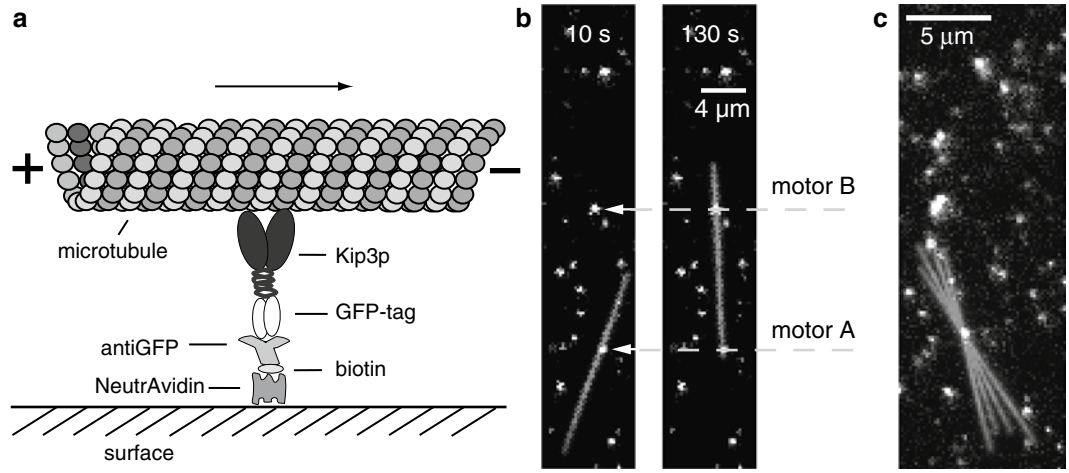


Fig. 1. Sandwich geometry of motor surface attachment for single-molecule gliding assay. (**a**) Schematic of the experiment. (**b**) A single surface attached Kip3p (budding yeast kinesin-8, motor A, *white*) transports a microtubule (*grey*). At a later time point (130 s), the microtubule binds to a second surface attached motor (motor B) and is transported by both motors. (**c**) Maximum projection of all frames while the microtubule was transported by a single molecule. The microtubule swivelled around a single attachment point which coincides with a single GFP-tagged Kip3p molecule.

3. Bind microtubules to the surface of the flow cell channel.
4. Bind protein of interest via the antibodies onto microspheres for motility experiment.

*3.3.1. Coat Microspheres Passively with NeutrAvidin*

1. Clean 10 µl of microspheres (Polysciences, USA, diameter ≈0.5 µm, 2.6 wt%) three times by centrifugation in 1 ml water (2 min, 16,000×*g*).
2. After the last centrifugation step, resuspend pellet in 100 µl PBS followed by a short sonication step.
3. Add while sonicating 500 µl of 0.2 mg/ml NeutrAvidin in PBS (see Note 6) to the microsphere solution.
4. Incubate for 4 h at 4°C while shaking in a thermomixer at 700 rpm.
5. Fill microspheres up to a volume of 1.5 ml with wash buffer A, split the volume into three times 500 µl.
6. Centrifuge at 5,300×*g* for 10 min with a bench top centrifuge (cooled to 4°C optional).
7. Resuspend pellets in 500 µl wash buffer A (dissolve the pellets by sucking the solution in and out with a pipette, squirting the solution onto the pellet followed by 5 s sonication).
8. Repeat steps 6 and 7 four times.
9. Centrifuge again, remove supernatant and keep 20 µl in the tube. Resuspend the pellets in these 20 µl (see step 7).
10. Collect the three bead solutions in a new tube and fill up to a final volume of 100 µl with wash buffer A.

11. Check if microspheres are clustered!
12. Cleaned NeutrAvidin-coated microspheres can be stored for several months at 4°C.

*3.3.2. Bind Biotinylated AntiGFP to Functionalized Microspheres (see Notes 6–9)*

1. Add 40 µl of wash buffer A to 10 µl of cleaned, biotin-binding protein-functionalized microspheres (diameter≈0.5 µm, 0.25 wt% (see Note 5) or use purchased microspheres).
2. Add 50 µl of 0.1 mg/ml biotinylated antiGFP (see Note 6) to the diluted microspheres from step 1 (mix while sonicating!).
3. Incubate at 4°C for 4 h while shaking in a thermoshaker with 700 rpm.
4. Fill microsphere solution up to a volume of 1.5 ml with wash buffer B (see Note 8).
5. Incubate 30 min.
6. Centrifuge at 5,300×*g* for 10 min with bench top centrifuge (cooled to 4°C, optional).
7. Resuspend pellets in 500 µl wash buffer A (dissolve the pellets by sucking the solution in and out with a pipette, squirting the solution onto the pellet followed by 5 s sonication).
8. Repeat steps 6 and 7 four times.
9. Centrifuge again, remove supernatant, and keep 20 µl in the tube. Resuspend the pellets in these 20 µl each (see step 7).
10. Collect the three bead solutions in a new tube.
11. Check if beads are clustered (see Note 10).

*3.3.3. Bind Microtubules to the Surface of the Flow Cell Channel*

Use filter paper or vacuum to flow given volumes through the channel of the flow cell while always keeping solution in the channel.

1. 20 µl tubulin antibody (diluted in PBS); incubate 10 min.
2. 20 µl PBS (to wash out unbound antibodies).
3. 20 µl F-127 (1% in PBS); incubate 10 min.
4. 5×20 µl BRB80.
5. 20 µl microtubules; incubate 10 min.
6. 20 µl motility buffer (to equilibrate channel).

*3.3.4. Motility Experiment*

1. Mix microspheres with GFP-tagged motor protein; incubate for 5–10 min.
2. Dilute microspheres in motility buffer and flow the solution into the channel with the microtubules.
3. The motility of microspheres along microtubules can be visualized by LED-illuminated video-enhanced DIC (Fig. 2) (11) (see Note 11).

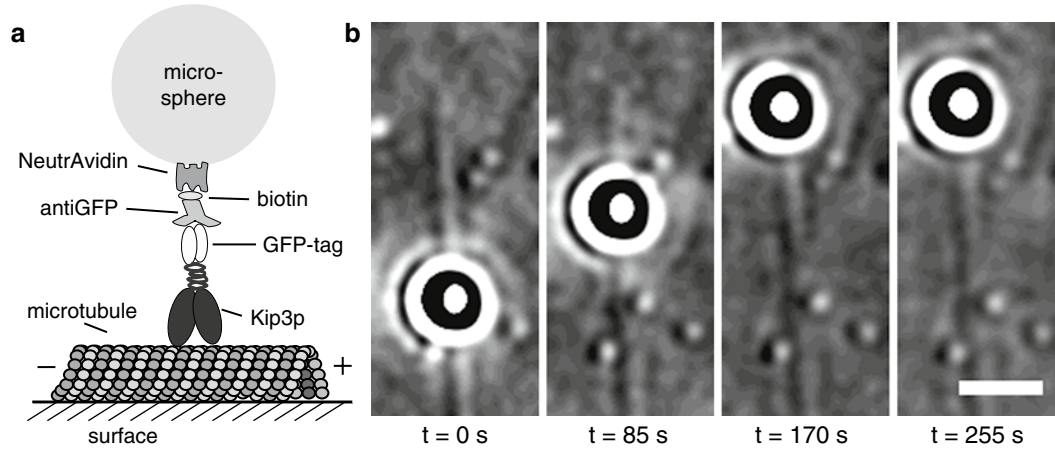


Fig. 2. Sandwich attachment for single-molecule microsphere assay. (**a**) Schematic of the experiment. (**b**) Time series of Kip3p-EGFP transporting an attached microsphere along a microtubule. The microsphere and microtubule are imaged using our video-enhanced LED-DIC (11). The microsphere stops at the microtubule end without detaching from the microtubule, which is characteristic for Kip3p (2). The scale bar corresponds to 2 μm.

## 4. Notes

1. Avidin, streptavidin, or NeutrAvidin can be used as biotin-binding protein. Note that they have different isoelectric points and thus a different net charge in your buffer of choice.
2. Streptavidin-coated microspheres can be purchased, for example from Bangs Laboratories (Fishers, USA), Polysciences (Warrington, USA), or Spherotech (Lake Forest, USA). Spherotech also sells functionalized microspheres of diameter < 0.5 μm.
3. Problems with purchased microspheres: First, purchased beads, especially with diameter < 1 μm are often strongly clustered. Break clusters by sonicating the microspheres with a tip sonicator for 30 s in ice (a bath sonicator is often not powerful enough to break the clusters) or by creating shear forces by passing many times through a 27¾ g needle. Second, often the microsphere suspension still contains free biotin-binding proteins and the biotin-binding capacity varies strongly. Wash the beads before use and test their functionality by binding them, for example, to biotinylated microtubules in kinesin-gliding assays (8). Microspheres should bind easily by diffusion exclusively to biotinylated microtubules and not to the surface.
4. We worked with polystyrene microspheres purchased form Spherotech and with polystyrene microspheres that we functionalized by passive adsorption of NeutrAvidin. A detailed protocol for the passive adsorption is given in the main text.

5. Problems with passive adsorbed NeutrAvidin: First, proteins are not covalently bound to the microsphere surface but can unbind slowly especially when changing the buffer conditions. Second, the strength of the adsorption depends on the surfactants used by the supplier. Third, if the protein detaches slowly from the surface, it is difficult to separate the microspheres from the biotin-binding protein (see Note 10).
6. Use five to ten times more protein than necessary to form a protein monolayer on the microsphere surface (see Bangslabs TechNote 204 for surface-binding capacity of various proteins http://www.bangslabs.com/literature/technotes).
7. You can upscale the amount of beads used in this protocol according to the amount of available antibody. It is easier to work with larger amounts due to loss of microspheres on the tube surface during the washing steps.
8. The biotin in the wash buffer B saturates the remaining free biotin-binding sites of the NeutrAvidin. If concentrations of biotin are too high, biotin will compete the biotinylated antibodies off the microsphere!
9. We used a monoclonal antibody against GFP produced in our institute that we biotinylated with the EZ-Link NHS-PEO Solid Phase Biotinylation kit. Commercially available biotin-conjugated antibodies can also be used, for example the Penta-His Biotin-conjugated antibody from Qiagen.
10. Free biotin-binding proteins in the microsphere suspension lead to the formation of protein aggregates and microsphere clusters after the addition of biotinylated antibodies to the microsphere suspension (biotin-binding proteins have four biotin-binding sites, and the antibodies are biotinylated at multiple sites).
11. Functional attachment is controlled by comparing in vivo speeds and, in the case of the bead and gliding assays, we checked speeds against single-molecule TIRF data.

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