5 Advances in Surface-based Assays for Single Molecules

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ABSTRACT

Optical trapping assays have stringent requirements for the controlled attachment of cytoskeletal filaments and motors to glass coverslips and polymeric colloidal spheres, whereas single-molecule fluorescence assays require surfaces that bind cytoskeletal filaments but repel fluorescent dyes. Many single-molecule assays have traditionally relied on fortuitous nonspecific interactions between motors, filaments, and surfaces, but extension to new motors has proven difficult, limiting the application of these techniques to new classes of actin- and microtubule-based motors. In this chapter, we present an overview of the physical principles driving surface interactions and a survey of practical surface treatments, with an emphasis on novel polymer-based surface chemistries that allow robust, stereospecific attachment of motors and filaments while inhibiting undesirable nonspecific interactions. Common protocols are included at the end of the chapter.

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INTRODUCTION

Members of the kinesin, dynein, and myosin superfamilies are essential for processes as diverse as cellular transport, cell division, muscle contraction, and melanosome migration. To accomplish

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these disparate tasks, many of these motors use the energy of ATP hydrolysis to generate force and translocate in discrete steps along cytoskeletal filaments. Various kinesins and myosins share a common architecture, with a cargo-binding tail connected through a stalk region to a conserved catalytic domain that binds both nucleotides and the cytoskeletal filament (Kull et al. 1996). In contrast, dyneins are significantly larger, with a microtubule-binding stalk and six linked AAA-ATPase (ATPases Associated with various cellular Activities) domains arranged in a ring (Marx et al. 2005). Diverse evolutionary pressures have also led to functional differences: Kinesins and dyneins move along microtubules, whereas myosins move along actin filaments; kinesins remain tightly bound to the filament in the presence of ATP, whereas ATP causes myosins and dyneins to detach (Romberg and Vale 1993); and kinesins and myosins contain only a single ATP-binding site, whereas dynein is capable of binding and hydrolyzing ATP at multiple binding sites in a cooperative manner (Kon et al. 2004; Takahashi et al. 2004). Even within a family, there is considerable diversity, with individual kinesin and myosin family members moving in either direction (Walker et al. 1990; Svoboda et al. 1993; Endow and Waligora 1998; Mehta et al. 1999; Wells et al. 1999) along filaments as monomers (Okada et al. 1995), dimers (Block et al. 1990; Zhang and Hancock 2004), or even higher-order assemblies (Cole et al. 1994; Klopfenstein et al. 2002; Kapitein et al. 2005).

Single-molecule techniques allow the observation and characterization of individual motors as they move along their linear substrates. Optical trapping assays have been used to track the position of single motors with high resolution, making possible measurements of both the fundamental step size and the average number of consecutive steps taken before dissociation (the "mechanical processivity") (Svoboda et al. 1993; Finer et al. 1994; Uyeda et al. 1996; Guilford et al. 1997; Mehta et al. 1999; deCastro et al. 2000; Purcell et al. 2002; Tominaga et al. 2003; Mallik et al. 2004; Rock et al. 2005; Toba et al. 2006; Valentine et al. 2006). Moreover, optical traps can be used to apply controlled loads, allowing determination of the maximal force a motor can produce or move against (Finer et al. 1994; Visscher et al. 1999; Block et al. 2003; Mallik et al. 2004; Toba et al. 2006). In the case of processive motors that take multiple steps before detachment, optical traps can be used to elucidate the force dependence of the kinetic cycle. Probing these force-dependent transitions, which are inaccessible using traditional solution biochemical techniques, has led to the development of detailed mechanistic models of motility for conventional kinesin, the mitotic kinesin Eg5, and several unconventional myosins (Block et al. 2003; Altman et al. 2004; Purcell et al. 2005; Veigel et al. 2005; Valentine et al. 2006). Single-molecule fluorescence assays also provide position information and can be used to monitor both the relative motion of motor domains during the stepping cycle and the oligomerization state of the protein (Vale et al. 1996; Sosa et al. 2001; Forkey et al. 2003; Yildiz et al. 2003, 2004; Okten et al. 2004; Ross et al. 2006; Toba et al. 2006). A combination of these two techniques has been used to probe the coupling of the biochemical cycle (monitored by the arrival of a single fluorescently labeled ATP) to the mechanical step of an individual myosin-II motor (Ishijima et al. 1998) and shows promise for further studies of additional motor proteins.

In all cases, single-molecule assays require controlled attachment of proteins to surfaces. Fortuitous nonspecific interactions are sometimes sufficient; however, more complex surface treatments and attachment schemes to control surface interactions and prevent nonspecific sticking are often required. In this review, we highlight common assay geometries and surface chemistries for in vitro single-molecule measurements of actin- and microtubule-based motor proteins. We emphasize novel polymer-based surface treatments that allow robust, stereospecific attachment of motors and filaments while suppressing nonspecific interactions, and we provide detailed tips and protocols for experimenters undertaking this work.

Optical Trapping Assays

Many excellent reviews provide comprehensive descriptions of optical trapping theory and instrument construction (Svoboda and Block 1994a; Visscher and Block 1998; Neuman and Block 2004); we provide only a brief description here. Optical traps are typically formed by a

high numerical aperture (NA) microscope objective that focuses a powerful laser beam to a diffraction-limited spot in the specimen plane. When an approximately micron-sized dielectric particle is brought near this focus, it experiences a force that can be separated into two components: the gradient force and the scattering force. The gradient force, which is proportional to the spatial gradient of the laser intensity, pulls the dielectric particle toward the center of the beam focus. The photon-scattering force, arising from the radiation pressure exerted by the incoming photons and proportional to the laser intensity, pushes the particle along the direction of beam propagation. For collimated laser beads that slightly overfill the back aperture of a microscope objective with NA ≥ 1.3, the gradient force dominates, and particles are held stably in three dimensions.

Single motor proteins can be attached to dielectric colloidal beads and stably trapped, allowing both high-resolution positional tracking and the application of controlled loads (Block et al. 1990, 2003; Visscher et al. 1999). Processive motors can be monitored over multiple kinetic cycles using an experimental geometry in which a single bead-attached motor is held in the optical trap and placed near a cytoskeletal filament bound to the coverslip (Fig. 5-1A). Nonprocessive motors require a different geometry employing two optical traps (a "three-bead assay") (Finer et al. 1994): The cytoskeletal filament is attached at either end to two beads held in separate optical traps while motor proteins are attached at low density to a platform (often another particle) adhered to the coverglass surface (Fig. 5-1B). The suspended filament is then positioned near the

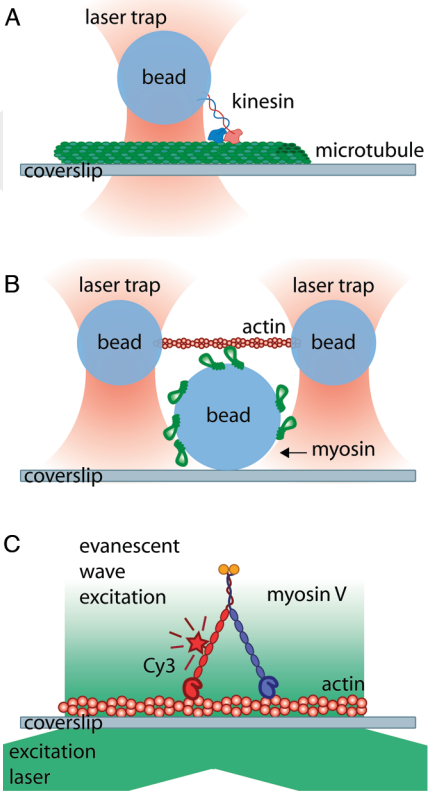

FIGURE 5-1. Examples of single-molecule assay geometries. (*A*) Single-bead optical trapping assay for kinesin. An optical trap is used to bring a bead-attached kinesin near a surface-bound microtubule (*B*) Three-bead optical trapping assay for myosin II. An actin filament is attached at each end to beads held in separate optical traps and suspended over a myosin II-coated coverslip-attached bead that serves as a pedestal. (*C*) Single-molecule fluorescence assay for myosin V. A single myosin V motor with a Cy3 label on a calmodulin subunit of one motor head walks along an actin filament adhered to the coverslip surface. Evanescent wave excitation penetrates a short distance into solution to excite the Cy3, and emission is detected by a low-light camera.

platform, allowing the motor proteins to make transient interactions with the filament while the two optical traps are used to monitor motor binding and measure filament displacement. The techniques described in this review focus on the single bead assay geometry, in which the filament is attached to the coverslip surface, but the principles are easily extended to other assays.

Single bead optical trapping experiments must meet the following requirements: (1) The motor protein must be securely attached to the bead for accurate measurements of position and force. In addition, for initial demonstrations of processivity, the bead must contain excess motor-binding sites, as such demonstrations require statistical fits to the fraction of beads that move over a range of motor concentrations, from less than one to multiple motors per bead. (2) Filaments must be firmly attached to the coverslip surface to prevent load-induced slippage and suppress thermal fluctuations of the substrate that could be erroneously interpreted as motor motion. Such thermal fluctuations can also inflate measurements of enzyme stochasticity, a tool used to determine the number of rate-limiting steps within the kinetic cycle (Schnitzer and Block 1995; Shaevitz et al. 2005). (3) Motor-coated beads must not adhere to the coverslip surface, as even transient surface interactions between the bead and the coverslip can lead to misinterpretations of motor motion.

Single-Molecule Fluorescence Assays

Several recent reviews provide detailed descriptions of fluorescence theory and experimental techniques (Peterman et al. 2004; Wazawa and Ueda 2005). Briefly, in single-molecule fluorescence assays, a fluorescent dye molecule is excited by the absorption of a photon from an excitation laser, and then returns to its low-energy ground state through the emission of a longer-wavelength (and lower-energy) photon. Because the emitted photons are spectrally distinct from those produced by the excitation laser, they may be isolated through optical filtering and detected with high sensitivity. Single-molecule fluorescence assays typically employ total internal reflection fluorescence (TIRF) microscopy, in which the excitation laser beam is directed at an oblique angle onto the interface between the glass flow cell and the aqueous specimen, either through the objective or by a prism. For angles of incidence greater than the critical angle, the light is totally internally reflected at the interface, and an evanescent wave propagates into solution (Fig. 5-1C). The intensity of the evanescent field decays exponentially with distance from the interface, limiting the depth of the excitation volume and increasing the signal-to-noise ratio for surface-localized fluorophores.

As with optical trapping assays, several constraints must be satisfied in single-molecule fluorescence experiments: (1) A recombinant protein is often required. The protein of interest is either fused to a member of the green fluorescent protein (GFP) family or a biotin tag, or is mutated to remove native surface-exposed cysteines and introduce new cysteines at sites targeted for labeling with maleimide-conjugated fluorophores. For studies of chemomechanical coupling, fluorescently labeled nucleotides are required. (2) The cytoskeletal substrate must be firmly attached to the coverslip surface, as thermal fluctuations of the filament can interfere with accurate tracking of labeled proteins. (3) Dye-labeled proteins and nucleotides must be repelled from the coverslip surface. Nonspecific sticking of dyes increases background signal, making protein tracking difficult, and can masquerade as the interaction of interest.

SURFACE COATINGS MODULATE THE INTERFACIAL ENERGY

Undesired adsorption of proteins and protein-coated beads to surfaces, as well as protein-mediated bead aggregation, can be a significant obstacle to the development of new single-molecule assays. Surface coatings are commonly used to alter the interfacial energy of the protein-surface boundary in order to reduce nonspecific adsorption. Although the complete calculation of all interfacial energies in a system is rarely feasible, a qualitative understanding of the dominant surface interactions in single-molecule assays is critical to selecting the proper coating agents.

Nonionic Interactions

Hydrophobic interactions are a strong nonionic contribution to the protein–coverslip and protein–bead interfacial energies (Israelachvili 1992). Proteins contain both hydrophobic (water-hating) and hydrophilic (water-loving) amino acid residues. Because the entropic penalty for solvation of hydrophobic regions is so high, the equilibrium folded conformation typically buries the hydrophobic residues in the internal core of the protein, while the hydrophilic residues extend out into solution. Some surfaces can destabilize this folded conformation by allowing hydrophobic portions of the protein to adhere and spread, excluding the interstitial water; the probability of such surface-induced denaturation increases with increasing surface hydrophobicity. A second nonionic force is the van der Waals attraction, arising from dipole–dipole interactions and dispersion forces among polarizable molecules. Although ubiquitous, van der Waals interactions are negligible in most applications due to electrostatic repulsion at short distances (Russel et al. 1989; Israelachvili 1992).

Surface stickiness arising from hydrophobic interactions is most easily overcome by pretreating the surface with a coating layer to increase its hydrophilicity and decrease the likelihood of the subsequent unfolding of the protein of interest (Valagao Amadeu do Serro et al. 1999). One common technique involves coating glass coverslips or glass or polymeric beads with a layer of globular sacrificial proteins ("blocking proteins") to create a self-assembled hydrophilic film. These proteins include bovine serum albumin (BSA), casein, and even commonly used oxygen-scavenging agents such as glucose oxidase and catalase. Alternatively, nonionic surfactants (such as Tween 20) may be used. The use of such amphiphilic molecules with high surface affinity has several advantages: Reagents are inexpensive and commercially available, and the protocol is easy to execute.

Nonetheless, incorporating blocking proteins or surfactants has limitations. Most importantly, formation of a stable hydrophilic film is sensitive to surface cleaning procedures and buffer conditions, reducing reproducibility. Moreover, because these blocking proteins and surfactants are not covalently attached to surfaces, they desorb over time and may be displaced by motor proteins with unusually high surface affinity. The use of casein, in particular, can be problematic for the determination of phosphate-dependent ATPase rates, as casein can contain contaminating phosphate (Schief et al. 2004). Despite these drawbacks, blocking-protein systems have been used extensively for studies of conventional kinesin (Kuo and Sheetz 1993; Svoboda and Block 1994b; Higuchi et al. 1997; Uemura et al. 2002; Block et al. 2003; Kawaguchi et al. 2003), unconventional myosins (Nishikawa et al. 2002; Uemura et al. 2004; Clemen et al. 2005), and dynein (Wang et al. 1995; Sakakibara et al. 1999; Kojima et al. 2002; Mallik et al. 2004; Ross et al. 2006). They are extremely useful as a first attempt at "quick and dirty" assay development.

A second approach to increase hydrophilicity involves coating the surface with covalently coupled or nonspecifically adsorbed nonionic polymers. Polyethylene glycol (PEG) is particularly good at reducing protein affinity and has been used widely to suppress protein binding in biomedical and research applications (Prime and Whitesides 1991; Benesch et al. 2001; Bhadra et al. 2002). The repulsive nature of PEG-coated surfaces has been attributed to steric effects arising both from the loss of conformational entropy of the polymer as the chains are compressed and from the energetically costly desolvation of the polymer by the impinging protein. Covalent attachment of bifunctionalized PEGs with a terminal N-hydroxysuccinimidyl ester to amine-coated surfaces is possible, preventing desorption with time or applied load. However, relatively lengthy incubation times are often required, reducing experimental throughput.

Copolymer systems that provide an attractive alternative to covalent coupling have recently emerged. These copolymers contain a large block of PEG that extends into solution to prevent protein adsorption, and a second type of polymer that is attracted to the coverslip surface, allowing self-assembly at the interface without covalent attachment. One class of PEG-based copolymers, commercially known as Pluronics (available from BASF Performance Chemicals), consists of polyethylene-PEG-polyethylene blocks (Amiji and Park 1992). The polyethylene (PE) blocks are strongly hydrophobic and adhere to many surfaces; pretreating the coverslip with a silanizing

agent (e.g., dimethyldichlorosilane) can increase the hydrophobic attraction. A second class of PEG-based copolymers, such as poly(L-lysine)-PEG graft copolymers (available from SurfaceSolutionS), relies on electrostatic interactions for self-assembly (VandeVondele et al. 2003). The positively charged poly-L-lysine (PLL) is attracted to the negatively charged clean glass coverslips, while the charge-neutral PEG chains extend into solution.

Whether covalently bound to, or self-assembled at, the coverslip or bead surface, PEG polymers are extremely efficient protein repellents. Polymer density can be well controlled, and it is possible to obtain or produce PEG-based compounds with pendant or terminal functional groups that allow the specific attachment of filaments or motor proteins to the coverslip or bead surface. The main drawback of using PEG-based polymer coatings is the expense of purchasing costly polymers. We have also found that dye-labeled nucleotides tend to adhere to PEG-coated surfaces, perhaps because the nucleotides are small enough to permeate the PEG layer without causing significant deformation or desolvation of the chains. Finally, unbound polymer must be washed out in order to prevent bead aggregation due to depletion effects (Asakura and Oosawa 1954; Tuinier et al. 2003).

Electrostatic Interactions

Charge-mediated interactions between proteins and surfaces also contribute to the interfacial energy. The surface of a typical protein contains both positively and negatively charged residues, leading to complex interactions with charged bead or coverslip surfaces. Counterions in buffers used for single-molecule assays "screen" the surface charge at large separations, giving rise to an electrostatic interaction free energy that decays exponentially with distance from the surface. The characteristic length of this decay, known as the Debye screening length, λ_D, varies inversely with the square root of the counterion concentration; λ_D is ~1 nm for a 100 mM monovalent salt solution (Israelachvili 1992). Thus, simply increasing the salt concentration reduces the distance over which electrostatic forces contribute to the interfacial energy and can reduce charge-mediated sticking if the electrostatic interactions are net attractive. Changes in pH (particularly near the isoelectric point for a protein) can also affect the interaction energy by modifying the charge of exposed residues on the protein. Modulating surface interactions by varying salt concentration and pH is inexpensive and easy to implement; however, global changes in ionic strength or pH can also affect motor dynamics. Alternatively, surface-attached charge-neutral polymer chains can be used to sterically exclude charged proteins from within a few Debye screening lengths of the surface. PEG-based coatings are particularly advantageous, since both undesirable charge-mediated and hydrophobic interactions are reduced.

Another approach takes advantage of electrostatic interactions by using polyelectrolyte-multilayer (PEM) coatings. In this case, surfaces are incubated with alternating layers of positively and negatively charged polymers to modify the interaction potential. By varying polymer type, incubation time, pH, the total number of layers, and the charge of the final layer, it is possible to create a wide variety of attractive or repulsive surfaces (Decher 1997). PEM coatings are a particularly good choice for repelling dye-labeled nucleotides, which carry a strong negative charge and tend to stick to both blocking protein layers and PEG-coated surfaces. By depositing alternating positively charged polyethyleneimine and negatively charged poly(acrylic acid) (PAA) layers, and ending with a final PAA layer, it is possible to create a highly negatively charged surface that is particularly effective at repelling dye-labeled nucleotides (Kartalov et al. 2003). PEM-coated surfaces are easy to create using commercially available, inexpensive polymers, and some (such as PAA) can be easily functionalized for specific attachment of filaments or motor proteins. Unlike dye-labeled nucleotides, most proteins have a heterogeneous surface charge density, allowing them to interact with a charged PEM-coated surface even when the net protein charge and the surface charge are of the same sign (Ladam et al. 2002). Unfortunately, these interactions can result in the nonspecific—and frequently undesired—adsorption of filaments or motor-coated beads.

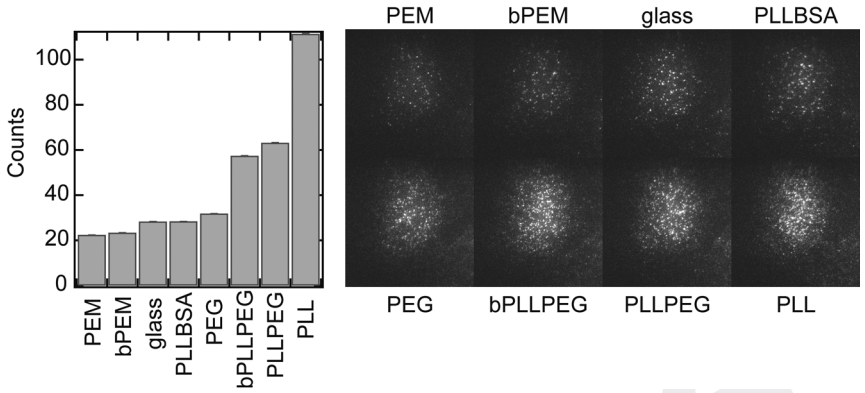

FIGURE 5-2. Repulsion of 100 nM Cy3-ATP suspended in 80 mM PIPES buffer from glass coverslips by different surface treatments. All surfaces have been plasma cleaned in air at 2 Torr for 5 minutes prior to surface treatment. (PEM = polyelectrolyte multilayers with PAA as final layer; bPEM = PEM surface with final PAA layer biotinylated; glass = clean glass; PLLBSA = poly-*L*-lysine-treated coverslips coated with 10 mg/ml of BSA; PEG = methoxyPEG-NHS esters covalently coupled to aminosilanized glass; bPLLPEG = biotinylated PLL-PEG copolymers; PLLPEG = PLL-PEG copolymers; PLL = poly-*L*-lysine-treated glass). Bar graph on left shows the average number of counts recorded in 10 msec by an avalanche photodiode for each surface. Pictures on right are typical fields of view for a cooled EMCCD camera (100 msec integration time, maximum gain, at –80°C).

Characterization of the Surface Affinity of Proteins and Nucleotides

Surface interactions are complex and difficult to predict. Consequently, optimizing surface treatments for a particular application requires the ability to quickly characterize the affinity of proteins or nucleotides of interest to a variety of coverslip and bead surfaces. To quantify adsorption of dye-labeled proteins or nucleotides to coverslips, we commonly visualize surfaces using TIRF microscopy and an electron-multiplying charge-coupled device (EMCCD) camera (standard tools of single-molecule imaging) and quantify dye adsorption by using an avalanche photodiode to count photons emitted by surface-bound dyes (Fig. 5-2). For nonfluorescent protein-coated beads used in optical trapping assays, it is possible to quantify adsorption by using conventional microscopy to measure the number of attached beads per field of view (Fig. 5-3).

In addition to measuring the affinity of proteins and nucleotides to surfaces, quantifying the number of active binding sites present on a bead is often crucial to determining the efficiency of coupling reactions and for troubleshooting assay conditions. This can be accomplished by incubating beads with excess protein, then centrifuging them to separate protein-coated beads from unbound proteins in the supernatant. The amount of protein remaining in the supernatant can then be quantified by measuring intrinsic fluorescence or using a traditional protein concentration assay (such as the Bradford or Lowry assay).

TECHNIQUE OVERVIEW

Preparing Clean Glass Coverslips and Slides

Commercially available "precleaned" coverslips are coated with organic residues that are visible under either differential interference contrast (DIC) light microscopy or fluorescence microscopy. Removal of these contaminants is crucial for precise control of the interactions between proteins or polymers and the surface of the microscope flow chamber. Additionally, cleaning procedures impart a reproducible negative charge to glass surfaces, facilitating the subsequent adsorption of positively charged layers through electrostatic interactions. Although cleaning coverslips alone is

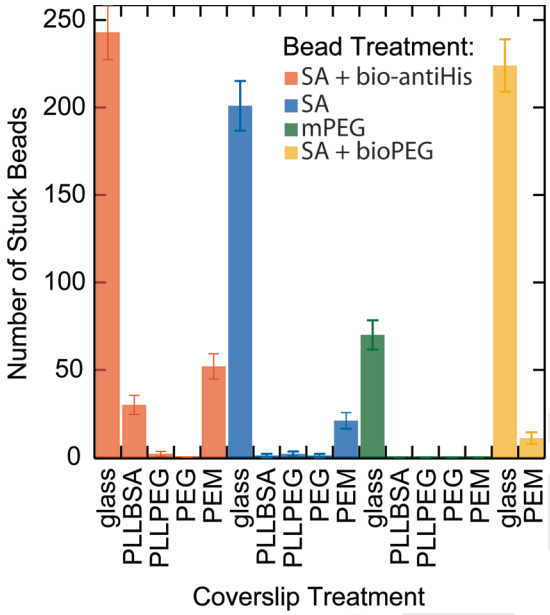

FIGURE 5-3. Protein adsorption for different combinations of bead and surface treatments. Each bar represents the number of stuck beads over 40 fields of view. (Coverslip treatments: glass = plasma-cleaned glass; PLLBSA = poly-*L*-lysine-treated glass coated with a layer of 10 mg/ml BSA; PLLPEG = glass coated with PLL-PEG copolymers; PEG = aminosilanized glass coated with a layer of covalently coupled PEG-NHS esters; PEM = polyelectrolyte multilayers. Bead treatments: SA = latex beads with covalently coupled streptavidin; SA+bio-antiHis = biotinylated anti-pentaHis antibody coupled to SA beads; mPEG = methoxy-PEG-NHS esters covalently coupled to amine beads; SA+bioPEG = SA beads incubated with biotinylated methoxyPEG chains). PEG polymers block protein adsorption more effectively than traditional blocking proteins, although in many cases blocking proteins are sufficient. PEM multilayers are not particularly effective at repelling protein adsorption; however, the addition of PEG chains to beads in this case can reduce bead sticking.

sufficient for most optical trapping applications, cleaning glass slides (as well as coverslips) can help reduce background for single-molecule fluorescence experiments.

Contaminated outer layers of glass can be removed through chemical etching, using various combinations of aggressive solvents (such as acetone, acetic acid, concentrated potassium hydroxide in water or ethanol, or mixtures of sulfuric acid and hydrogen peroxide in water). Alternately, surface-bound contaminants can be removed by exposure to a gas plasma discharge. Plasma cleaning requires the purchase of a commercial plasma cleaner, but avoids the use of harsh chemicals and saves time: Plasma cleaning takes roughly 5 minutes, whereas chemical etching can take an hour or more.

Substrate–Surface Attachments

Nonspecific attachments (Fig. 5-4A–C)

Microtubules and actin filaments have net negative charge, giving rise to electrostatic repulsion from the negatively charged coverslip surface. Despite this repulsion, microtubules have occasionally been directly adsorbed onto clean glass (Block et al. 1990; Carter and Cross 2005). In most cases, either the coverslip surface or the cytoskeletal filaments themselves are first modified in order to promote adsorption through either electrostatic or hydrophobic interactions. Coverslip modifications include aminosilanization (Coy et al. 1999) or the deposition of positively charged polypeptide coatings (e.g., PLL) (Schnitzer and Block 1997; Block et al. 2003; Mallik et al. 2004) to promote electrostatic attraction of microtubules, and silanization (Svoboda and Block 1994b;

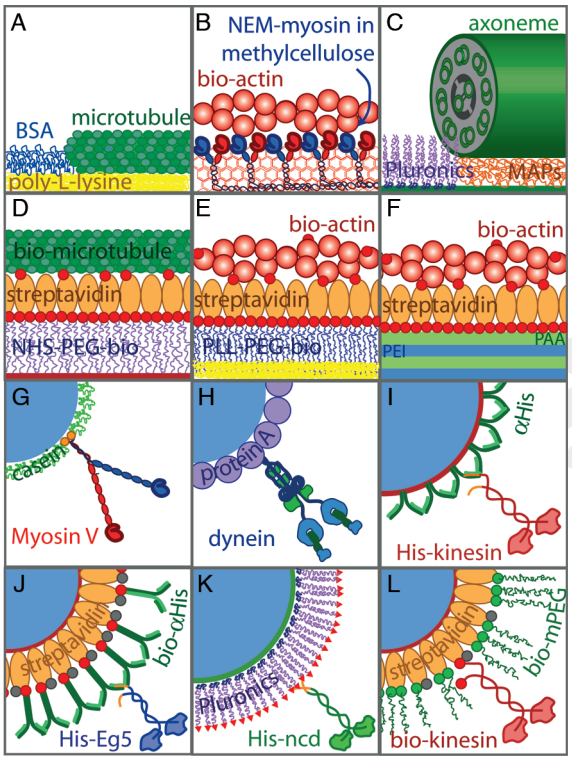

FIGURE 5-4. Examples of possible attachment schemes for linking cytoskeletal filaments to coverslips (*A*–*F*) and motors to beads (*G*–*L*). (*A*) Microtubules nonspecifically bound to a poly-*L*-lysine surface surrounded by BSA to prevent further protein adsorption. (*B*) Biotinylated actin filaments bound to the surface through NEM-treated myosin molecules that are permanently in rigor. The myosins are embedded within a methylcellulose layer. (*C*) Axoneme nonspecifically attached to bare glass and surrounded by Pluronics PEG-based copolymers. (*D*) Biotinylated microtubules bound through a streptavidin linkage to bifunctional biotinylated PEG chains that have been covalently coupled to the glass surface. (*E*) Biotinylated actin filaments bound to biotinylated PLL-PEG block copolymers through a streptavidin linkage. (*F*) Biotinylated F-actin filaments bound to a biotinylated PAA layer of a PEM surface through a streptavidin linkage. (*G*) Myosin V motor nonspecifically attached to carboxylated latex bead in the presence of casein. (*H*) Cytoplasmic dynein bound to carboxylated latex bead in the presence of protein A. (*I*) His-tagged kinesin bound to a covalently coupled F′-ab antibody fragment. (*J*) His-tagged Eg5 bound to commercial streptavidin beads through a biotinylated anti-pentaHis antibody. (*K*) His-tagged ncd bound to silanized silica beads through modified Pluronic copolymers containing a nickel-chelating group (NTA). (*L*) Biotinylated kinesin and biotinylated methoxy-PEG chains bound to a commercial streptavidin bead.

Coy et al. 1999; deCastro et al. 1999) or the deposition of a layer of nitrocellulose (Syed et al. 2006) to facilitate hydrophobic interactions between the glass and microtubules or actin filaments. Alternatively, accessory binding proteins can be nonspecifically adsorbed to the coverslip; these accessory proteins then bind to cytoskeletal filaments, attaching them firmly to the glass surface. In this manner, microtubules have been bound to nonspecifically adsorbed antitubulin antiserum (Wang et al. 1995), F-actin filaments have been bound to N-ethylmaleimide (NEM)-treated myosin II motors in a nitrocellulose layer (Clemen et al. 2005; Warshaw et al. 2005), and purified axonemes have been adsorbed through their microtubule-associated proteins (MAPs) (Vale et al. 1996; Romberg et al. 1998; Sosa et al. 2001; Yildiz et al. 2004). Finally, fluorescently labeled cytoskeletal filaments are often adsorbed directly to clean glass (Higuchi et al. 1997; Kojima et al. 1997, 2002; Sakakibara et al. 1999; Inoue et al. 2001; Seitz et al. 2002; Uemura et al. 2002; Kawaguchi et al. 2003; Okada et al. 2003; Lakamper and Meyhofer 2005; Ross et al. 2006; Seitz and Surrey 2006; Toba et al. 2006), likely through a combination of electrostatic and hydrophobic interactions.

Specific attachments (Fig. 5-4D–F)

Microtubules and actin filaments can also be attached to the surface specifically, most commonly by using biotin–avidin linkages. Biotinylated F-actin filaments and biotinylated microtubules can be polymerized from mixtures of commercially available unmodified and biotinylated actin monomers and tubulin heterodimers, respectively (Uemura et al. 2004). Polymerized filaments must be purified from free subunits (Pardee and Spudich 1982) to prevent biotinylated subunits from rapidly diffusing to the surface and saturating available avidin-binding sites (Valentine et al. 2006). These purified biotinylated filaments may be attached directly to nonspecifically adsorbed neutravidin (Rief et al. 2000; Rock et al. 2000) or to a biotinylated surface through a multivalent streptavidin linkage. A variety of options are available to create this biotinylated surface: Biotinylated BSA (Uemura et al. 2004; Churchman et al. 2005; Syed et al. 2006) or biotinylated casein (Nishikawa et al. 2002) may be nonspecifically adsorbed to the coverslip; bifunctionalized PEG, end-terminated with both a biotin group and an NHS ester, may be covalently coupled to aminosilanized glass (Rasnik et al. 2004); biotinylated-PEG-PLL copolymers are available (Valentine et al. 2006); and PAA layers of PEM coatings may be biotinylated by covalently coupling amino–polyethylene oxide–biotin oligomers (commercially available as an EZ-Link kit from Pierce) to the pendant carboxylated groups of the polyelectrolyte using water-soluble-carbodiimide (WSC)-based chemistry (Kartalov et al. 2003).

Motor–Bead Attachments

Nonspecific attachments (Fig. 5-4G,H)

As with substrate-surface attachments, many schemes exist to couple motor proteins to beads, and occasionally nonspecific attachments are feasible. Native kinesin proteins purified from squid optic lobes (Block et al. 1990; Visscher et al. 1999) or bovine brain (Higuchi et al. 1997; Nishiyama et al. 2001), recombinant kinesin heavy chains expressed in the presence and absence of the associated light chains (Coy et al. 1999), cytoplasmic and flagellar dyneins (Wang et al. 1995; Sakakibara et al. 1999; Kojima et al. 2002; Mallik et al. 2004), and myosin V (Rief et al. 2000; Rock et al. 2000; Uemura et al. 2004; Clemen et al. 2005) have all been bound nonspecifically to silica or carboxylated polystyrene beads in the presence of blocking proteins. In addition, a recombinant kinesin family member (ncd) construct fused to glutathione-*S*-transferase (GST) has been adsorbed nonspecifically to silanized silica beads (Allersma et al. 1998).

In other cases, motors are attached through a nonspecifically adsorbed binding partner. Antibodies raised against the carboxy-terminal peptide of myosin have been nonspecifically adsorbed to carboxylated polystyrene beads and used as a specific attachment point for myosin motors (Tominaga et al. 2003), and dynein has been anchored to polystyrene beads precoated with Protein A crosslinked to quantum dots for fluorescence visualization (Toba et al. 2006). Generally, negatively charged colloids (such as carboxylated polystyrene or silica spheres) bind motor proteins, MAPs, and actin-binding proteins (ABPs) better and are less likely to interact with negatively charged cytoskeletal filaments than positively charged colloids (such as amino-functionalized polystyrene). Nonspecific attachments are easy to implement and do not require extensive modifications to the motor; however, nonspecifically adsorbed proteins may detach under the high forces applied by an optical trap.

Specific attachments (Fig. 5-4I–L)

When a recombinant protein expression system producing soluble tagged motors is available, motors can be specifically attached to beads. Such specific attachments position motors in a known and functional orientation and allow the application of high forces, as bond rupture forces are both well-characterized and large. Fortunately, most myosin and kinesin family motors possess

relatively long stalk domains that extend far from the catalytic core, allowing truncation and modification to introduce a biotin or epitope tag. Such tags also permit the efficient purification of expressed motor proteins through affinity chromatography.

Motor proteins expressed in *Escherichia coli* can be biotinylated through the attachment of either the full biotin carboxyl carrier protein (BCCP) (Cronan 1990) or a minimal 14-amino acid peptide from the BCCP protein (Beckett et al. 1999) to the end of the stalk domain; posttranslational modification during expression results in the transfer of biotin to a specific lysine residue. Monofunctional maleimide esters of biotin can also be covalently coupled to surface-exposed cysteines (Lakamper and Meyhofer 2005), although such coupling frequently requires the creation of "cysteine-light" mutant proteins containing only a single surface-exposed cysteine to ensure that motors are bound in a known orientation. Once biotinylated, motor proteins may be attached to avidin-, streptavidin-, or neutravidin-coated beads for optical trapping studies (Berliner et al. 1994). Such beads are commercially available from Bangs Laboratories and Spherotech, among others, or they can be made through the covalent coupling of avidin molecules to carboxylated microspheres using standard WSC coupling chemistries.

Several specific attachment schemes based on antibody interactions are also available. Antibodies to GFP, FLAG, myc, GST, and pentaHistidine (His) (Okada et al. 2003) tags are all commercially available and can be covalently coupled to beads for attachment of tagged motors. PentaHistidine (His) tags require the addition of only five additional amino acids, and they can also be attached to avidin beads through a biotinylated anti-pentaHistidine antibody (Asbury et al. 2003; Valentine et al. 2006), or to surfaces coated with Pluronic copolymers that have been chemically modified to include the nickel-chelating group nitrilotriacetic acid (NTA) (deCastro et al. 1999).

For particularly sticky proteins, it may be necessary to attach PEG polymers directly to beads in addition to coating the coverslip. Biotinylated proteins can be attached to avidin beads and then incubated with bifunctionalized PEG polymers end-terminated with both a methoxyl and a biotin group. The bifunctionalized polymers saturate remaining biotin-binding sites, creating a polymer layer that resists surface adsorption. Similarly, for His-tagged proteins, it is possible to couple a mixture of biotinylated anti-pentaHis antibodies and bifunctionalized PEG polymers to avidin beads: These beads resist surface adsorption and bind His-tagged motors, albeit with a significantly reduced binding capacity. In principle, bifunctionalized PEG polymers, end-terminated with a biotin group and an NHS-ester, may be covalently coupled to amine-functionalized beads. Subsequent adsorption of a layer of avidin molecules to the biotinylated PEG layer should provide a mechanism for attachment of biotinylated motor proteins. However, although similar biotin–avidin–biotin linkages work well to bind filaments to PEG-coated coverslips, in our hands the PEG layer prevents efficient binding of avidin to beads, making this coupling scheme unusable.

Single-molecule fluorescence experiments require the coupling of fluorescent dye molecules or quantum dots to individual proteins. As with bead coupling, it is occasionally possible to couple fluorescent dyes to motor-binding partners, avoiding functional changes to the motors themselves. For calmodulin-carrying myosin motors, fluorescently labeled calmodulin can be exchanged for endogenous calmodulin to label myosin head domains (Forkey et al. 2003; Yildiz et al. 2003). Similarly, cytoplasmic dynein associated with dynactin containing four fluorescently labeled dynamitin subunits can be purified from transgenic mice expressing dynamitin with an enhanced GFP (EGFP) fused to the carboxyl terminus (Ross et al. 2006). Most proteins, however, require modification prior to labeling. Truncated motor constructs containing only a single surface-reactive cysteine can be fluorescently labeled through the covalent coupling of maleimide esters of fluorescent dyes, although most motors require the creation of a "cysteine-light" mutant (as mentioned earlier) whose surface-exposed cysteines have been mutated to other residues (Inoue et al. 2001; Sosa et al. 2001; Yildiz et al. 2004; Churchman et al. 2005; Lakamper and Meyhofer 2005). Biotinylated motor proteins can be bound to streptavidin conjugated to quantum dots (Warshaw et al. 2005; Seitz and Surrey 2006) or labeled with single fluorophores. Both

biotin and GFP (Nishikawa et al. 2002; Seitz et al. 2002) tags offer the advantage that they can be used to visualize the motion of individual proteins during single-molecule fluorescence experiments or to couple motors to beads for optical trapping measurements.

SUMMARY

A large variety of protein- and polymer-based surface chemistries have now been developed to aid in single-molecule assay development. Many methods are robust and modular, requiring only biotin- or epitope-tagged motors, but not relying on specific motor properties. By mixing and matching various attachment schemes, experimenters now have an expanded toolkit with which to tackle new classes of motor proteins, as well as complex macromolecular assemblies of cytoskeletal proteins.

Protocol 1

Cleaning of Glass Coverslips and Slides

This protocol describes two methods for cleaning glass coverslips and slides, using either chemicals or plasma cleaning.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Ethanol (300 ml, 0.2-µm filtered if used for fluorescence assays)
H_2O (0.2-µm filtered) (Milli-Q or similar)
Potassium hydroxide (KOH) pellets (100 g) <!>

Equipment

Beakers
Container with lid for storing coverslips
Coverslips or microscope slides (glass)
Nitrogen gas (filtered, compressed) <!>
Oven preset to ~45°C
Plasma Cleaner (Harrick Plasma PDC-001, or similar) (for plasma cleaning method)
Teflon or glass racks (glass or ceramic racks for plasma cleaning method)
Ultrasonic cleaners, tabletop (2; Branson 1510, or similar)

METHOD

For chemical cleaning, follow Steps 1–9. For plasma cleaning, follow Steps 10–15.

Chemical Cleaning

1. Add 100 g of KOH pellets to a beaker filled with 300 ML of ethanol. Stir vigorously to dissolve as much KOH as possible. Once dissolved, the solution should turn slightly brown. Place the KOH-filled beaker in an ultrasonic cleaner and de-gas for 5 minutes.

   Careful: This is extremely concentrated!

2. Fill two more beakers with 300 ML of H_2O and de-gas each for 5 minutes, leaving the second H_2O-filled beaker in the ultrasonic cleaner.
3. Place coverslips and slides in Teflon or glass racks.
4. Submerge the rack of coverslips (or slides) in the KOH-filled beaker and sonicate for 5 minutes.

5. Remove the rack from the KOH solution and rinse by dipping it up and down in an H_2O-filled beaker, breaking the air–H_2O interface with each dip. Continue until the H_2O runs smoothly off the glass without beading.
6. Submerge the rack of coverslips (or slides) in the other H_2O-filled beaker and sonicate for 5 minutes.
7. Rinse the rack under a stream of H_2O for 30 seconds or more, taking care to rinse each slide or coverslip thoroughly.
8. For optical trapping measurements, dry the rack in a hot (~45°C) oven, and store clean coverslips in closed container.
9. For single-molecule fluorescence studies, store the rack under filtered H_2O in a closed beaker for up to 1 week. Dry in a laminar flow box using vacuum under a stream of filtered, compressed nitrogen gas immediately before use.

Replace the rinse H_2O after about 5 racks of coverslips or slides.

Plasma Cleaning

10. Place glass coverslips or slides in ceramic or glass racks.
11. Place the rack in the center of the plasma cleaner chamber.
12. Start the vacuum pump.
13. For plasma cleaners with a flow mixer, adjust air intake until the chamber pressure equilibrates to 2 Torr and expose the coverslips (or slides) to plasma discharge with power setting at "high" for 5 minutes.
14. For plasma cleaners without a flow mixer, expose coverslips (or slides) to plasma discharge with power setting at "high" for 5 minutes, opening the pump valve to bleed in a small amount of air and increase the plasma intensity about every minute.
15. For single-molecule fluorescence studies, use cleaned coverslips (or slides) immediately or store the rack under filtered H_2O in a closed beaker for up to 1 week.

AQ1

Protocol 2

Flow Cell Assembly Using PLL-coated Coverslips and Preparation of BSA-coated Coverslips

This protocol describes the preparation of PLL-coated coverslips, the assembly of flow cells using these coverslips, and subsequent coating of the coverslips with BSA or biotinylated BSA.

MATERIALS

Reagents

Bovine serum albumin (BSA; Calbiochem)
If a biotinylated surface is required, substitute biotin-BSA (Vector Labs) for BSA.
Ethanol
PEM80 (80 mM PIPES [pH 6.9], 1 mM EGTA, and 4 mM $MgCl_2$)
Poly-L-lysine (PLL) (Sigma)

Equipment

Container with lid for storing coverslips
Coverslips (clean; rectangular for flow cell assembly)
Double-sided sticky tape
Filters (Millipore, Steriflip)
Oven preset to ~45°C
Pipette tip
Razor blade
Slides (clean for single-molecule fluorescence)
Teflon or glass rack

METHOD

Preparation of PLL-coated Coverslips

1. Add about 500 µl of PLL to 400 ML of ethanol. Stir for 2 minutes.
2. Place coverslips in a Teflon or glass rack and submerge the rack in PLL solution. Incubate for 10 minutes (without stirring).
3. Rinse the rack under a stream of H_2O for 30 seconds or more, taking care to rinse each slide or coverslip thoroughly.
4. Dry the rack in a hot (~45°C) oven, and store clean coverslips in a closed container.

   The "stickiness" of PLL-coated coverslips tends to vary. If microtubules appear wiggly or if motor-coated beads adhere to the coverslip surface, decrease or increase the PLL concentration as needed.

Flow Cell Assembly

5. Place two pieces of double-sided sticky tape across a slide about 3 mm apart to form a channel.
6. Use a razor blade to remove overhanging tape.
7. Place a rectangular coverslip across the slide so that the long axis of the coverslip is aligned with the long axis of the channel formed by the tape, and the coverslip edges stick out from the slide to provide a platform for buffer exchanges.

   Use clean coverslips or, if BSA coating will follow, PLL-coated coverslips prepared in Steps 1–4.
8. Use a plastic pipette tip to press down on the coverslip surface, eliminating any air bubbles in the tape.
9. Exchange buffer through the channel using either filter paper or vacuum suction.

Preparation of BSA-coated Coverslips

10. Add 20 mg of BSA to 2 ml of PEM80 and slowly mix to dissolve BSA; avoid foaming.
11. Filter BSA solution using 0.2-μm filters.
12. Flow about 25 μl of BSA solution into flow cell chamber (assembled in Steps 5–9 with PLL-treated coverslips). Incubate for >10 minutes.
13. Flow through about 100 μl of PEM 80 to remove excess BSA.

    The amount of BSA that is necessary to prevent surface adsorption depends on the application. For surfaces that are too sticky or not sticky enough, increase or decrease the BSA concentration as necessary.

Protocol 3

Preparing Covalently Coupled PEG-coated Coverslips

This protocol describes the preparation of covalently coupled PEG-coated coverslips, and a modification for biotinylated PEG-coated surfaces.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Acetone<!>
Methoxy-polyethylene glycol, with a terminal N-hydroxysuccinimide (mPEG-SPA; Nektar)

If a biotinylated PEG-coated surface is required, replace the pure mPEG-SPA polymer with an admixture of mPEG-SPA and Biotin–PEG–NHS (Nektar).

Sodium bicarbonate (100 mM [pH 8.7]; prepare fresh)
Vectabond (Vector Labs)

Equipment

Coverslips (clean)
Double-sided sticky tape
Nitrogen gas (filtered) <!>
Slides

METHOD

1. Aminosilanize coverslips as follows:
   i. Dissolve 7 ml of Vectabond reagent in 350 ml of acetone. Mix well.
   ii. Submerge coverslips in pure acetone and incubate for 5 minutes.
   iii. Transfer coverslips to Vectabond solution and incubate for 5 minutes.
   iv. Submerge coverslips in H_2O, and gently dip for about 30 seconds to remove excess silanizer.
   v. Dry coverslips under vacuum-filtered nitrogen in a laminar flow box for about 5 minutes.
2. Assemble flow cells using dry aminosilanized coverslips, slides, and double-sided sticky tape (see Protocol 2).
3. Dissolve mPEG-SPA at about 12.5% w/v in 100 mM sodium bicarbonate.
4. Flow about 25 µl of mPEG-SPA solution into the chamber and incubate at room temperature for 2–4 hours.
5. Rinse away excess polymer by flowing through at least 100 µl of 100 mM sodium bicarbonate.

Protocol 4

Preparing PLL-PEG-coated Coverslips

This protocol describes the preparation of PLL-PEG-coated coverslips and a modification for biotinylated PLL-PEG-coated surfaces. It is adapted from instructions provided by SurfaceSolutions.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Phosphate-buffered saline (PBS; 8 mM sodium hydrogen phosphate, 2 mM potassium dihydrogen phosphate, 3 mM potassium chloride, and 137 mM sodium chloride [pH 7.4]; 0.2-µm filtered)

Poly-L-lysine-*graft*-polyethylene glycol (PLL-PEG ; SurfaceSolutions)

If a biotinylated PEG-coated surface is required, replace the pure PLL-PEG with a biotinylated copolymer (SurfaceSolutions).

Tris-B buffer (10 mM Tris, 10 mM NaCl [pH 8.0]; 0.2-µm filtered)

Equipment

Coverslips (clean)
Double-sided sticky tape
Slides

METHOD

1. Assemble flow cell using clean coverslips and slides (see Protocol 2)
2. Prepare PLL-PEG at 1 mg/ml diluted in PBS.

   Store PLL-PEG solution in small aliquots at –20°C for later use if needed.
3. Flow through about 30 µl of PLL-PEG prepared in Step 2, and incubate for 30 minutes (or more).
4. Wash out by flowing through 100 µl of Tris-B buffer.

Protocol 5

Preparing PEI-PAA-multilayer-coated Coverslips for Use with Dye-labeled Nucleotides

This protocol, adapted from Kartalov et al. (2003), describes the preparation of multilayer-coated coverslips for use with dye-labeled nucleotides. It also includes a method for biotinylation of polyelectrolyte-coated coverslips.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Alconox detergent <!>
1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) <!> (for biotinylation)
EZ-Link Amine-PEO-Biotin Labeling kit (Pierce) (for biotinylation)
Hydrochloric acid (HCl) <!>
2-[N-morpholino]ethanesulfonic acid (MES) <!> (for biotinylation)
Polyacrylic acid (PAA) (Sigma)
Polyethyleneimine (PEI) (Sigma) <!>

Equipment

Beakers (4, glass, 1000-ml)
Coverslips (clean), loaded into Teflon or glass racks
Filters (0.2 μm)
Nitrogen gas (filtered) <!> (for biotinylation)
Rotating platform
Ultrasonic cleaner (tabletop)
Watch glasses (4, large)

METHOD

Preparation of PEI-PAA-multilayer-coated Coverslips

1. Prepare Alconox solution as directed by manufacturer.
2. Clean all beakers and watch glasses in Alconox solution in the ultrasonic cleaner for 15 minutes. Rinse well with H_2O and air-dry.
3. Make polymer solutions by adding 50 ml of H_2O to 0.5 g of PEI and 50 ml of H_2O to 0.5 g of PAA. Slowly dissolve at room temperature by gentle rotation for 30 minutes or more.
4. Once dissolved, dilute each solution to 2 mg/ml (final concentration), adjust each to pH 8.0 with HCl, and 0.2-μm filter.

5. Fill one clean beaker with PEI solution, one with PAA, and two with H_2O (one for rinsing and one for storage). Cover each with a clean watch glass.
6. Immerse the rack of coverslips in PEI solution for 12 minutes. Remove excess polymer by immersing in H_2O-filled beaker and gently swirling 30–40 times (without breaking air–H_2O interface).
7. Immerse in PAA solution for 12 minutes. Remove excess polymer as in Step 6.
8. Repeat Steps 5 and 6 to deposit a total of 4 polymer layers (two negatively charged and two positively charged).
9. Store polyelectrolyte-coated coverslips inside an H_2O-filled beaker.
10. For single-molecule fluorescence, use coverslips within 1 day of preparation. Make stock solutions fresh weekly and store them at 4°C. Rewash beakers every several months.

Biotinylation of Polyelectrolyte-coated Coverslips

11. Prepare MES-B (10 mM MES [pH 5.5], 0.2-μm filtered)
12. Dry polyelectrolyte-coated coverslips under filtered nitrogen in laminar flow box.
13. Assemble flow cells as in Protocol 2 using dry polyelectrolyte-coated coverslips and clean slides.
14. Prepare EDC solution by dissolving 48 mg of EDC in 5 ml of MES-B for a final concentration of 50 mM.
15. Immediately prepare BB by combining 10 μl of EDC solution, 10 μl of Bio-LC-PEO-Amine from EZ-Link kit, and 80 μl of MES-B.
16. Flow about 20 μl of BB into each flow chamber and incubate for at least 30 minutes at room temperature.
17. Rinse away excess coupling reagents by flowing through at least 100 μl of MES-B.

Protocol 6

Preparing Unmodified Microtubules and Attaching Them to a PLL Surface

This protocol describes the preparation of unmodified microtubules (MTs) and a method for attaching them to a PLL surface.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Bovine serum albumin (BSA)
Dimethyl sulfoxide (DMSO) <!>
Guanosine triphosphate (GTP; 10 mM in PEM104; Sigma)
PEM80 (80 mM PIPES [pH 6.9], 1 mM EGTA, and 4 mM $MgCl_2$)
PEM104 (103.6 mM PIPES [pH 6.9], 1.3 mM EGTA, and 6.3 mM $MgCl_2$)
Sodium azide, 65 g/L <!>

Careful: This is extremely toxic.

Taxol <!> (Paclitaxel; 10 mM in DMSO <!>)
Tubulin minus glycerol (10 mg/ml; Cytoskeleton)

Equipment

Ice
Incubator preset to 37°C
Poly-L-lysine (PLL)-treated coverslips
Slides
Vortex mixer

METHOD

Preparation of Unmodified MTs

1. Make PEM/GTP by combining 15.4 µl of PEM104 and 2.0 µl of 10 mM GTP.
2. On ice, combine 15.2 µl of PEM/GTP and 2.2 µl of DMSO. Vortex, then add 4.8 µl of 10 mg/ml tubulin minus glycerol.
3. Incubate at 37°C for 1 hour to polymerize MTs.
4. During polymerization, prepare the stabilization buffer (STAB) by combining the following:

   306.9 µl of PEM80

   45 µl of 10 mM GTP

   42.3 µl of 65 g/L sodium azide

10.8 µl of 10 mM Taxol

45 µl of DMSO

5. After 1 hour of incubation, stabilize the MTs by adding 2.0 µl of STAB.
   Stabilized MTs should be stable at room temperature for several months.

Attaching Unmodified MTs to a PLL Surface

6. Assemble a flow cell with a slide and a PLL-treated surface (see Protocol 2).
7. Prepare PEMTAX by adding 1 µl of 10 mM Taxol to 1 ml of PEM80.
8. Dilute the stock solution of MTs from Step 5 roughly 100-fold in PEMTAX, and flow through 25 µl of dilute MTs into each flow chamber. Incubate at least 30 minutes.
9. Prepare 10 mg/ml BSA dissolved in PEMTAX. Flow through 100 µl of this solution to wash out unbound MTs and to coat surfaces with a layer of sacrificial protein. Incubate at least 10 minutes before use.

Protocol 7

Preparing Biotinylated Microtubules and Attaching Them to a Biotinylated Surface

This protocol describes the preparation of biotinylated microtubules (bMTs) and a method for attaching them to a biotinylated surface.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Biotin (100 mM in DMSO <!>)
Biotinylated surface: any of the following:
  biotinylated-BSA (see Protocol 2)
  biotinylated-PEG (see Protocol 3)
  biotinylated-polyelectrolyte-multilayer (PEM) (see Protocol 5)
Biotinylated tubulin (lyophilized; Cytoskeleton)
Dimethyl sulfoxide (DMSO) <!>
Guanosine triphosphate (GTP; 10 mM in PEM104; Sigma)
PEM80 (80 mM PIPES [pH 6.9], 1 mM EGTA, and 4 mM $MgCl_2$)
PEM104 (103.6 mM PIPES [pH 6.9], 1.3 mM EGTA, and 6.3 mM $MgCl_2$)
Sodium azide, 65 g/L <!>
  Careful: This is extremely toxic.
Streptavidin (Prozyme)
Sucrose
Taxol <!> (Paclitaxel; 10 mM in DMSO <!>)
Tris-B buffer (10 mM Tris, 10 mM NaCl [pH 8.0]; 0.2-μm filtered)
Tubulin minus glycerol (10 mg/ml; Cytoskeleton)

Equipment

Centrifuge tubes
Ice
Incubator preset to 37°C
Ultracentrifuge (Beckman Coulter Optima, with MLS-50 rotor, or similar)
Vortex mixer

METHOD

Preparation of bMTs

1. Make PEM/GTP by combining 2.47 ml of PEM104 with 0.32 ml of 10 mM GTP.
2. On ice, resuspend 20 μg of lyophilized biotinylated tubulin in 4 μl of cold PEM/GTP for a final concentration of 5 mg/ml.

3. On ice, combine 3.8 µl of 5 mg/ml biotinylated tubulin with 15.4 µl of 10 mg/ml tubulin minus glycerol.
4. Mix 60.8 µl of PEM/GTP with 8.8 µl of DMSO and vortex. Add 19.2 µl of tubulin mixture and mix well.
5. Incubate at 37°C for 1 hour to polymerize bMTs.
6. During polymerization, prepare the stabilization buffer (STAB) by combining the following:

   306.9 µl of PEM80

   45 µl of 10 mM GTP

   42.3 µl of 65 g/L sodium azide

   10.8 µl of 10 mM Taxol

   45 µl of DMSO
7. After polymerization, add 2 µl of STAB to the polymerized bMT mixture to stabilize bMTs. Store bMTs at room temperature overnight.
8. Prepare a sucrose cushion (2 ML of PEM/GTP, 220 µl of DMSO, 200 µl of STAB, and 400 Mg of sucrose). Layer polymerized bMTs on top of it. Centrifuge at 26,000*g* in an MLS-50 rotor for 20 minutes at 30°C.
9. Remove the supernatant and resuspend the pellet in 100 µl of STAB.
10. Store bMTs at room temperature (for several weeks if needed). Dilute approximately 50- to 100-fold immediately before use in optical trapping assays.

Attaching bMTs to a Biotinylated Surface

11. Assemble a flow cell with a biotinylated surface (see Protocol 2). Wash away any unbound biotinylation reagents by flowing through 100 µl of Tris-B.
12. Prepare streptavidin at 0.2 mg/ml in Tris-B. Flow through 30 µl of this solution and incubate for at least 10 minutes.
13. Flow through 100 µl of Tris-B solution to wash away any unbound streptavidin.
14. Prepare PEMTAX by adding 1 µl of 10 mM Taxol to 1 ml of PEM80. Flow through 100 µl of PEMTAX to exchange buffers in preparation for deposition of bMTs.
15. Dilute stock solution of bMTs from Step 10 approximately 100-fold in PEMTAX, and flow through 25 µl of dilute bMTs into each flow chamber. Incubate at least 30 minutes.
16. Add 20 µl of 100 mM biotin to 1 ml of PEMTAX and flow through 100 µl of biotin-PEMTAX to remove free bMTs and quench any remaining biotin-binding sites on the surface-attached streptavidin proteins. Incubate for 5 minutes before use.

Protocol 8

Preparing Penta-His Antibody-coated Beads Using Biotin/Avidin Linkages

This protocol describes the use of biotin/avidin linkages to coat beads with penta-His antibody.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Biotin (100 mM in DMSO <!>) (optional; see Step 7.iii)
Biotin-conjugated penta-His antibody (Qiagen)
Biotinylated methoxy-polyethylene glycol (mPEG) (2 mg in 0.5 ml PEM80, Nektar) (optional; see Step 7.iii)
PEM80 (80 mM PIPES [pH 6.9], 1 mM EGTA, and 4 mM $MgCl_2$)
Sodium phosphate buffer (SPBT; 100 mM [pH 7.5], with 0.1% Tween 20)
Streptavidin-coated polymeric colloidal beads (Spherotech, 0.44 μm, or similar)

Equipment

Benchtop minicentrifuge
Ice
Microcentrifuge tubes
Rotating platform
Sonicator (ultrasonic homogenizer) with a micro cup horn attachment

METHOD

1. Mix 70 μl of PEM80 with 10 μl of streptavidin-coated beads in a microcentrifuge tube.
2. Fill the sonicator with H_2O and ice (to prevent heat-induced protein denaturation) and immerse the bottom of the tube containing the bead mixture in the cup. Sonicate for 2 minutes (or longer if beads appear "clumpy").
3. Add 20 μl of the biotin-conjugated penta-His antibody solution and mix well.
4. Gently rotate at room temperature for 1 hour.
5. Wash five times to remove excess antibody. For each wash, centrifuge bead mixture at 14,000 rpm for 6 minutes at 4°C, discard supernatant, and resuspend the pellet in an equal volume of SBPT. Pipette vigorously (without introducing bubbles) and scrape the bottom of the tube to fully resuspend the pellet.
6. Store the beads at 4°C on a rotator.

   Beads are typically active for approximately 3 months or until efficiency of binding His-tagged motor proteins to antibody-coated beads diminishes.

7. For use in optical trapping assays, proceed as follows:
   i. Sonicate antibody-coated beads and dilute by approximately an additional factor of 50.
   ii. Incubate beads with His-tagged motor proteins for 4–12 hours. Dilute motor protein concentration sufficiently to ensure that on average each bead carries only one motor protein (for more detail, see Svoboda and Block 1994b).
   iii. To prevent avidin-mediated adsorption to surfaces containing biotin–avidin linkages, saturate free biotin-binding sites by adding 10 µl of free biotin to each 200 µl of the bead–motor mixture during incubations. For particularly "sticky" proteins, substitute 10 µl of biotinylated mPEG.

Protocol 9

Preparing Covalently Coupled Antibody-coated Beads

This protocol describes the coating of carboxy-terminated polystyrene spheres with penta-His antibody using EDC-based coupling chemistry.

MATERIALS

CAUTION: See Appendix for appropriate handling of materials marked with <!>.

Reagents

Carboxy-terminated polystyrene spheres (such as 0.51-µm P(S/V-COOH) from Bangs Laboratories)
1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) <!>
Glycine
2-[N-morpholino]ethanesulfonic acid (MES buffer; 100 mM [pH 5.5]) <!>
Penta-His mouse monoclonal antibody (Qiagen)
Sodium phosphate buffer (100 mM [pH 7.5])
Sulfo N-hydroxysuccinimide (NHS)
Tween 20

Equipment

Benchtop minicentrifuge
Ice
Rotating platform
Sonicator (ultrasonic homogenizer) with a micro cup horn attachment

METHOD

1. Fill sonicator with water and ice. Sonicate 200 µl of stock bead solution for 2 minutes (or longer if clumps of beads are observed). **AQ2**
2. Add 500 µl of MES buffer to 100 µg of antibody.
3. Mix 20 mg of NHS, 1.3 ml of MES buffer, and 500 µl of antibody solution.
4. Add sonicated bead solution and invert several times to mix well.
5. Add 50 Mg of solid EDC to the bead solution and invert to mix well.

   For the beads we commonly use, this is roughly 400-fold molar excess of EDC to active surface-attached carboxyl groups.
6. Incubate on rotator overnight at room temperature.
7. Prepare 1 M glycine solution in 10 mM sodium phosphate buffer (pH 7.5). **AQ3**
8. Add 500 µl of 1 M glycine solution to the bead mixture and incubate for 15 minutes to quench any unreacted carboxyl groups.

9. Separate the beads into 5 equal fractions of 500 µl each.
10. Wash five times to remove excess antibody. For each wash, centrifuge the bead mixture at 14,000 rpm for 10 minutes at 4°C. After each centrifugation, discard the supernatant and resuspend the pellet in 200 µl of 100 mM sodium phosphate buffer, supplemented with 0.1% Tween 20, by vigorously pipetting up and down.
11. After the last wash, add 300 µl more sodium phosphate buffer to bring the total volume up to 500 µl.
12. Store the beads at 4°C on a rotator.

The beads are typically active for at least 6 months.

For more information on EDC-based coupling chemistry, see Hermanson (1996).

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AQ1 Do you dry the slides as in Step 9 of the chemical method?
AQ2 Is stock solution from manufacturer or do you prepare it?
AQ3 10-fold dilution of sodium phosphate listed in reagents, or should this step say 100 mm sodium phophate buffer?