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Quantum Dot Labeling Strategies to Characterize Single-Molecular Motors

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Abstract

Recent advances in single-molecule labeling and detection techniques allow high-resolution imaging of the motion of single molecules. Molecular motors are biological machines that convert chemical energy into mechanical work. Myosin Va (MyoVa) is a well-characterized processive molecular motor, essential for cargo transport in living organisms. Quantum dots (Qdots) are fluorescent semiconductor nanocrystals that are extremely useful for single-molecule studies in biological sciences. High-resolution video microscopy and single-particle tracking of a Qdot-labeled MyoVa motor molecule allow the detection of individual steps in vitro and in live cells.

Keywords

Single molecule; Myosin; Actin; Total Internal Reflection Microscopy; Single-Particle Tracking; Steps

1. Introduction

Video microscopy and single-particle tracking of fluorescently labeled molecules allow detection of the motion and stepping dynamics of single molecules of motor proteins. Quantum dots (Qdots) offer a number of advantages over traditional fluorescent dyes. Although approximately the same size as a GFP molecule, they are more than 20 times as bright and are extremely photo-stable, which allows long-term observation. Qdots also demonstrate very narrow fluorescence emission spectra, but very broad excitation spectra. This allows easy multiplexing of experiments using a single-excitation wavelength, and even the possibility of simultaneous observation of multiple colors. This property also allows selection of excitation and emission wavelengths that are very far apart (large Stokes shift), which reduces background autofluorescence in order to improve the signal-to-noise ratio. A large number of Qdot surface coatings are commercially available which allows for a variety of attachment strategies, both covalent and noncovalent.

Myosin Va (MyoVa) is an actin-based molecular motor that is involved in the short-range transport of a number of intracellular cargoes, including RNA, ER, melanocytes, and exocytic vesicles. A molecule of this motor comprises two heavy chains. Each heavy chain comprises an N-terminal motor domain that binds actin and hydrolyzes ATP, followed by a long neck domain consisting of 6IQ domains, where calmodulin light chains bind, while the C-terminal region forms the cargo-binding domain (CBD). In the absence of cargo, the C-terminal CBD interacts with the N-terminal motor domains to inhibit catalytic activity and motion (1). The motor moves along actin filaments in an alternating “hand-over-hand” mechanism, traveling 36 nm with each step. This distance matches the pseudo-repeat of the actin helix, allowing motion with minimal “spiraling” around the actin filament. The

catalytic cycle of each motor domain is sensitive to both forward and backward strains, a feature known as “gating,” which ensures that the two heads remain catalytically out of phase with each other. This aspect allows the motor to take many successive steps in the same direction along the actin filament without detaching, the hallmark of processive movement.

The tight coupling between the mechanical and hydrolytic processes of the MyoVa motor molecule makes it an ideal system to explore enzymatic activity through observation of a physical process. It is even possible to observe the individual steps of a single motor molecule, where each step is a readout of a single catalytic cycle of a single enzyme molecule. In vitro approaches allow easy manipulation of the environment, such as temperature, ionic strength, and substrate availability. This approach can also be applied to analyze the activity of MyoVa in the context of a cellular environment, where complexities of the cytoskeleton and cytosol complicate even the simplest aspects of this motor's activity.

2. Materials

The following stock reagents and buffers are common to all protocols presented in this chapter:

1. 1 M dithiothreitol (DTT) in dH₂O. Store in 0.5-mL aliquots at −20°C.
2. Bovine serum albumin (BSA): 10 mg/mL in dH₂O. Store in 100-μL aliquots at −20°C.
3. Actin buffer (AB) (10×): 250 mM imidazole, pH 7.4, 40 mM MgCl₂, 10 mM EGTA, 250 mM KCl. Store at 4°C.
4. Myosin buffer (10×): 250 mM imidazole, pH 7.4, 40 mM MgCl₂, 10 mM EGTA, 3 M KCl. Store at 4°C.
5. 0.1 M ATP disodium salt in dH₂O. Freeze in 100-μL aliquots at −20°C.

2.1. Protein: Quantum Dot Conjugation Strategies

2.1.1. MyoVa HMM to Streptavidin Qdot

1. Qdot 655 Streptavidin Conjugate (Invitrogen). Store at 4°C (see Note 1).
2. Biotinylated MyoVa heavy meromyosin (HMM): We use mouse MyoVa, truncated at amino acid 1098, which removes the C-terminal CBD and a part of coiled-coil rod domain to create an HMM construct. This construct is constitutively active in this assay. All constructs feature a C-terminal FLAG epitope for protein purification. For site-specific biotinylation, we use an 88 amino acid sequence segment from *E. coli* (2), which is biotinylated at a single lysine during expression in sf9 cells. Depending on the experiment, this sequence can be appended to the very N-terminus of the MyoVa sequence (termed N-bio) (3) or between the MyoVa and FLAG sequences (termed C-bio) (4).

2.1.2. Full-Length MyoVa to Carboxylated Qdot

1. Full-length MyoVa (FL-MyoVa) protein (5).
2. Qdot 655 ITK carboxyl quantum dots (Invitrogen).

¹In most of our experiments, we use Qdots which emit at 655 nm. But other Qdots, such as 565 nm, can be used and give similar results.

2.1.3. MyoVa HMM-YFP (N-Bio) to Antibody-Conjugated Qdot

1. MyoVa HMM-yellow fluorescence protein (YFP) (N-Bio): Truncated at amino acid 1098 and includes an N-terminal biotin tag and C-terminal YFP sequence (6).
2. Anti-GFP antibody: (α -GFP, mAb 3E6, Invitrogen). Dissolve dry antibody in 1× actin buffer with 10 mM DTT to a final concentration of 0.5 mg/mL. Store in 10- μ L aliquots at -20°C .
3. Qdot 655 goat F(ab')₂ anti-mouse IgG conjugate (H + L) (Invitrogen). Store at 4°C .

2.2. In Vitro Motility Assay

Many high-resolution imaging options are possible. We use Total Internal Reflection Fluorescence (TIRF) microscopy for its high signal-to-noise ratio. Our microscope configuration is as follows:

1. Nikon TE2000-U microscope equipped with a PlanApo (100×, 1.49 n.a.) objective lens configured for through-the-objective TIRF microscopy.
2. Excitation with argon laser (488-nm line).
3. Software-controlled emission filter wheel.
4. DVC-1412: GenIV intensified high-resolution 12-bit digital camera (DVC company, Austin, TX).

The following buffers and proteins can be prepared well in advance and will store well.

1. Creatine phosphate (CP, Sigma-Aldrich): 100 mM in dH₂O. Store in 100- μ L aliquots at -20°C .
2. Creatine phosphokinase (CPK). Store at -20°C .
3. Oxygen scavengers: Combine 175 mg glucose, 1.35 mg catalase, and 2 mg glucose oxidase. We recommend preparing multiple aliquots according to the above recipe in individual 1.7-mL microcentrifuge tubes. These tubes should be stored dry at -20°C .
4. Fluorescently labeled actin: Filamentous actin (F-actin) is purified from chicken pectoralis as described by Pardee and Spudich (7). For fluorescent labeling, 1 μ M F-actin is incubated overnight at 4°C in 1× actin buffer with 10 μ M DTT and either 1 μ M tetramethylrhodamine B isothiocyanate (TRITC) phalloidin or 1 μ M Alexa 660 phalloidin.
5. N-ethylmaleimide (NEM) myosin: NEM-modified myosin prepared as described by Warshaw et al. (8).

The following solutions are made immediately prior to performing the experiment. Due to the presence of the oxygen scavenging system in almost all components, they remain usable for about 2 h. If longer experimentation times are required, we recommend preparing fresh buffers as necessary.

1. Prepare 1× myosin buffer: Combine 1 mL of 10× myosin buffer and 100 μ L of 1 M DTT. Add enough dH₂O to make 10 mL.
2. Prepare oxygen scavengers: Dissolve one tube of oxygen scavengers with 600 μ L dH₂O. Mix by gently pipetting up and down. Be careful not to form bubbles or aerate the solution. Mixture is rather viscous (see Note 2). Keep on ice.

3. Prepare 1× actin buffer: Combine 1 mL of 10× actin buffer, 100 μ L 1 M DTT, 1 mg CPK, 100 μ L 0.1 M creatine phosphate, and 200 μ L oxygen scavengers (prepared fresh in item 2). Add enough water to make 10 mL. Keep on ice.
4. Prepare AB-BSA: Combine 50 μ L 10 mg/mL BSA with 450 μ L 1× actin buffer (from item 3). Keep on ice.
5. Prepare AB-BSA-ATP: Combine 50 μ L BSA, 940 μ L actin buffer, and 10 μ L from 100 mM ATP stock. Keep on ice.
6. Prepare fluorescent actin: Dilute 10 μ L TRITC phalloidin-labeled actin (1 μ M) with 100 μ L AB. Keep on ice (see Note 3).
7. Prepare NEM-myosin: Dilute NEM-myosin to 0.5 mg/mL in 1× myosin buffer. Keep on ice.
8. Prepare MyoVa:Qdot conjugate according to the protocol from Subheading 3.1.

2.3. In Vivo Motility Assay

1. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS).
2. Hypertonic loading buffer: DMEM, with 0.5 M sucrose, 10% w/v PEG 1000. Filter sterilize and store at 4°C.
3. Hypotonic lysis buffer: Combine 3 parts DMEM, 2 parts dH₂O. Filter sterilize and store at 4°C.
4. 35-mm glass bottom cell culture dish (MatTek, Ashland, MA).

3. Methods

3.1. Protein: Quantum Dot Conjugation

Qdots that are currently commercially available are all multivalent, meaning that they can bind multiple motor molecules. However, it is possible to conduct experiments on single molecules by carefully controlling the stoichiometry of mixing.

We have excellent results using Qdots and MyoVa in a 4 to 1 molar ratio. With these conditions, more than 77% of Qdots should have no motor bound, but remain silent in both the in vivo and in vitro conditions. Of Qdots that do have bound motor molecules (and are therefore observed in the assays), Qdots that have bound only a single motor molecule represent approximately 90% of the population, calculated using binomial equation (4).

There are a number of different strategies for conjugation of motor molecules to Qdots. The following are three different protocols that have proven successful with MyoVa.

3.1.1. MyoVa HMM to Streptavidin Qdot—The binding of streptavidin to biotin is among the strongest non-covalent interactions. Biotinylation of proteins can be done as a posttranslational modification in the host organism (1, 2), which allows for specific placement of the tag, or after protein purification in a more nonspecific manner (9).

²Oxygen scavengers reduce oxidative damage to proteins and photobleaching of organic fluorophores. This preparation retains function for about 2 h and cannot be refrozen or reused. In our experience, it is best to prepare fresh buffers if the experiment will run long instead of trying to “freshen up” the scavengers.

³To maintain long actin filaments, we find it best to handle actin preparations carefully to avoid shearing. This includes transferring actin preparations using wide bore tips or slicing off the terminal ~1 cm from a standard pipette tip.

This approach can be used to label either the N-terminal head (N-bio) or C-terminal tail domain (C-bio) of MyoVa HMM molecules. Using these conditions with the N-bio HMM construct, we observe 72-nm steps in the majority of trajectories (~97%). This indicates that most often, only one of two heads per molecule are bound by a Qdot. We rarely observe 36-nm steps (~3% of trajectories), which would be consistent with each head being bound with separate Qdots. If each head is bound with a different colored Qdot, it is possible to directly observe the hand-over-hand movement of MyoVa (3) (see Note 4). Qdots attached to the C-terminus of MyoVa show 36-nm steps, although the spatial resolution is reduced significantly, presumably due to the high flexibility in the coiled-coil tail domain.

1. Prepare 1× actin buffer: In a 15-mL conical tube, combine 1 mL of 10× actin buffer, 100 μ L 1 M DTT, add dH₂O to bring the volume to 10 mL. Keep on ice.
2. Prepare AB-BSA: Add 450 μ L 1× actin buffer to a 50 μ L 10 mg/mL BSA aliquot. Final BSA concentration is 1.0 mg/mL. Keep on ice.
3. Dilute both MyoVa HMM-YFP and Qdot 655 Streptavidin Conjugates to 200 nM in AB-BSA.
4. Mix 4 μ L of Qdots with 1 μ L of MyoVa HMM-YFP (a 4:1 molar ratio) and incubate for 10 min at room temperature (see Note 5).

3.1.2. Full-Length MyoVa to Carboxylated Qdot—Full-length MyoVa is typically considered catalytically inactive, with the C-terminal CBD sterically blocking the catalytic cycle of the heads (1, 10, 11). However, nonspecific binding of this myosin's CBD to carboxylated Qdots does result in apparent motion presumably by disrupting the head–CBD interaction of the inhibited state.

1. Prepare 1× actin buffer: In a 15-mL conical tube, combine 1 mL of 10× actin buffer, 100 μ L 1 M DTT, add dH₂O to bring the volume to 10 mL. Keep on ice.
2. Prepare AB-BSA (0.1 mg/mL BSA): Combine 990 μ L 1× actin buffer with 10 μ L 10 mg/mL BSA.
3. Dilute both full-length MyoVa and carboxy-Qdots to 200 nM in AB-BSA (0.1 mg/mL BSA).
4. Mix 4 μ L Qdots with 1 μ L FL-MyoVa and incubate for 10 min at room temperature.

3.1.3. MyoVa HMM-YFP to Antibody-Conjugated Qdot—Antibody-conjugated Qdots can be used to attach motor molecules in a specific manner, and are also suitable for single-molecule studies. It is important to use antibodies of high affinity (low K_d) for this application. This protocol describes conjugation of Qdot 655 antibodies to MyoVa molecules with a C-terminal YFP using an α -AFP monoclonal antibody.

1. Combine 2 μ L goat α -mouse IgG-conjugated Qdot 655 (1 μ M) with 2 μ L 3E6 α -GFP mAb (3.3 μ M).
2. Incubate for 60 min on ice.

⁴When labeling motor molecules where the motor domain is to be labeled, it is important to use limiting protein relative to the Qdots. If N-terminal domains of multiple motor molecules bind to the same Qdot, a situation analogous to a “three-legged race” ensues. However, when coupling motor molecules by their C-terminal or CBD, it is possible to use the Qdot as a cargo for transport by an ensemble of motor molecules. In this situation, it is possible to conjugate using a molar excess of myosin relative to the Qdots.

⁵We occasionally flick the tube with a finger to ensure mixing for better attachment of motors to Qdot.

3. During the above incubation, prepare 1× actin buffer: In a 15-mL conical tube, combine 1 mL of 10× actin buffer, 100 μ L 1 M DTT, add dH₂O to bring the volume to 10 mL. Keep on ice.
4. Dilute MyoVa HMM-YFP protein to 500 nM in 1× actin buffer.
5. Mix 4 μ L anti-GFP-coated Qdots (500 nM) with 1 μ L MyoVa HMM-YFP (500 nM).
6. Incubate for 30 min on ice.

3.2. In Vitro Motility Assay

In vitro experiments allow easy and controlled manipulation of experimental conditions. Confinement of actin tracks to the two-dimensional cover glass surface provides idealized imaging conditions, which allows for acquisition of very high-resolution data. A 10- μ L flow cell chamber as described previously (8) was used and the following solutions are introduced into the flow cell (see Note 6).

1. Flow 20 μ L NEM-myosin into the flow cell and incubate for 2 min. The NEM-myosin binds to glass surface and serves to fix actin filaments to the glass surface. This attachment produces much better results than adhering actin directly to the glass.
2. Wash the flow cell with 6 volumes (120 μ L) 1× actin buffer to remove excess NEM-myosin.
3. Introduce 20 μ L AB-BSA into the flow cell and incubate at room temperature for 2 min. This is a “blocking” step to reduce nonspecific binding of motors or Qdots to the glass surface.
4. Wash the flow cell with 100 μ L of 1× actin buffer.
5. Introduce 20 μ L of fluorescent actin into flow cell and incubate at room temperature for 2 min.
6. Wash the flow cell with 100 μ L 1× actin buffer.
7. Dilute Qdot-labeled MyoVa to 0.2–1.0 nM in AB-BSA-ATP (see Note 7).
8. Flow 20 μ L into the flow cell and observe under microscope at $25 \pm 1^\circ\text{C}$. (See an example of the results possible using this approach as shown in Fig. 1).

To observe the Qdot-labeled motors and fluorescently labeled actin, we use a Nikon TE2000-U microscope equipped with a PlanApo (100 $\times$, 1.49 n.a.) objective lens for through-the-objective total internal reflection fluorescence (TIRF) microscopy (5, 6, 12). Fluors are excited with 488-nm argon laser line. A software-controlled filter wheel is used to switch between emission filters specific for Qdots or actin filaments (see Note 8 regarding correction between two imaging channels). Images are captured by using a DVC-1412:GenIV intensified high-resolution 12-bit digital camera (DVC company, Austin, TX) at 166-ms integration time. We normally record 300–800 frames at 2–5 frames/s.

⁶We find that the most effective way to introduce solutions into the flow cells is to devise a support such that the flow cell is held at about 20° to the horizontal. Place the pipette tip with the solution right at the lip on the upper edge of the flow cell and hold a Q-tip at the lower lip of the flow cell. Capillary action quickly draws in the first solution. Subsequent additions are drawn into the flow cell by wicking action of the Q-tip.

⁷To measure the velocity and run length of MyoVa, we use saturating ATP (1–2 mM). To reduce the stepping rate to ~2 steps per second, we reduce the ATP concentration to 300 nM. As can be seen in Fig. 1, this allows better sampling and accuracy in order to observe the steps of the individual heads, also see ref. (3).

For image analysis and high-resolution tracking, we utilize custom-written software that fits the position of Qdot-labeled MyoVa with a two-dimensional Gaussian, which approximates the Qdot point-spread function with 6-nm accuracy (3, 12, 13). We also have excellent results with the automatic particle-tracking plug-in Spot Tracker 2D (14) for Image J (version 1.34s) digital image processing software (National Institutes of Health, Bethesda) and the step-fitting algorithm developed by Kerssemakers et al. (15). To measure the run length and velocity, a manual particle-tracking plug-in MTrack J is often used. This program determines the centroid position based on the image intensity with 20 nm resolution. The velocity is determined as the mean of a Gaussian distribution while run length distributions are fitted with $p(x) = Ae^{-x/\lambda}$ to determine the characteristic run length λ , where $p(x)$ is the relative frequency of the motor traveling a distance x along a track and A is a constant.

Although the protocol presented here is for the characterization of a single motor molecule, increasing the MyoVa:Qdot mixing ratio results in the creation of small ensembles of motor molecules all bound to the same Qdot cargo. This approach can be extended further to study the interaction of combinations of heterologous motors, such as that reported by Ali et al. (5).

3.3. In Vivo Motility Assay

Qdot-labeled MyoVa molecules can also be delivered into the cytosol of cultured mammalian cells in order to study the activity of these motors in a physiological environment. Although several approaches are available for delivery of protein:Qdot conjugates into the cytosol, we had excellent results using an osmotic shock-based protocol described by Okada and Rechsteiner (16) (see Note 9).

1. Plate COS-7 cells into a 35-mm glass bottom dish at a low density ($\sim 10^5$ cells) in 1.5 mL DMEM with 10% FBS and grow under standard tissue culture conditions (37°C, 5% CO₂) overnight.
2. Prepare MyoVa:Qdot complexes using a high-affinity attachment (i.e., biotin:streptavidin) as described (Subheading 3.1.1).
3. Dilute MyoVa:Qdot conjugates into 1.5 mL hypertonic loading buffer for a final concentration of 30 pM Qdots.
4. Prewarm buffers and media to 37°C in a water bath.
5. Aspirate media from cells and replace with MyoVa:Qdot conjugates in loading buffer from step 3.
6. Incubate for 10 min at 37°C.
7. Aspirate loading buffer.
8. Add 4 mL of hypotonic lysis buffer.
9. Incubate for 2 min at room temperature.

⁸Chromatic aberrations can cause significant errors when trying to relate the positions of two different color Qdots. To compensate for this, we create a “color correction matrix” by simultaneously imaging both imaging channels during a raster scan of multicolor fluorescent beads (Ultra Rainbow Fluorescent Particles, SpheroTech, Libertyville, IL). The discrepancy between the apparent coordinates of individual beads between the two imaging channels at different locations within the field of view is then used to populate an “offset matrix,” which is interpolated to contain a correction value for every pixel in the field of view. Simply adding or subtracting the appropriate correction value allows the observed coordinates from one color image to be aligned with the corresponding coordinates in the other color image.

⁹Internalization of Qdot-labeled MyoVa by this approach can be verified by labeling pinosomes by introducing 5 mg/mL FITC-Dextran 50k (Fluka) in the loading buffer and imaging prior to the hypotonic lysis step. Pinosomes appear as punctate structures, some of which colocalize with Qdots. Subsequent osmotic shock lyses the pinosomes, resulting in a diffuse haze throughout the cell.

10. Aspirate lysis buffer and add 1.5 mL DMEM.
11. Image immediately. An example of the MyoVa movements that can be detected with this approach is shown in Fig. 2.

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Fig. 1.

In vitro stepping of N-bio myosin Va. 72-nm steps are apparent for a MyoVa HMM molecule labeled with a Qdot at the N-terminus of one head. As expected, the motion is linear and unidirectional (*lower right inset*). Although the entire molecule moves with 36-nm steps, the labeled head demonstrates a 72-nm displacement for each step (*upper left inset*).

Fig.2.

In vivo stepping of N-bio myosin Va HMM. Qdot-labeled MyoVa HMM molecules delivered into cultured mammalian fibroblast cells (COS-7 cells) demonstrate random-looking trajectories (*upper left inset*). However, when imaged with high spatial (15 nm) and temporal (60 Hz) resolution, periods of linear movement can be detected (*boxed region in upper left inset, expanded in lower right inset*). When using a MyoVa construct that is labeled at its N-terminus, stepping behavior with the characteristic 72-nm step can be detected within these periods of linear movement.