Differential Labeling of Myosin V Heads with Quantum Dots Allows Direct Visualization of Hand-Over-Hand Processivity

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ABSTRACT The double-headed myosin V molecular motor carries intracellular cargo processively along actin tracks in a hand-over-hand manner. To test this hypothesis at the molecular level, we observed single myosin V molecules that were differentially labeled with quantum dots having different emission spectra so that the position of each head could be identified with ~6-nm resolution in a total internal reflectance microscope. With this approach, the individual heads of a single myosin V molecule were observed taking 72-nm steps as they alternated positions on the actin filament during processive movement. In addition, the heads were separated by 36 nm during pauses in motion, suggesting attachment to actin along its helical repeat. The 36-nm interhead spacing, the 72-nm step size, and the observation that heads alternate between leading and trailing positions on actin are obvious predictions of the hand-over-hand model, thus confirming myosin V's mode of walking along an actin filament.

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Myosin V is a double-headed molecular motor that delivers intracellular cargo over long distances by moving processively along actin filament tracks. Each head has a long neck, composed of six light chain binding motifs, that allows the myosin V to span the actin helical repeat while taking 36-nm steps. These steps are believed to occur in a hand-over-hand fashion where the heads alternate between ''leading'' and ''trailing'' positions (Fig. 1). To date there is no direct evidence for this model, but it has been inferred from studies in which the polarization (1) or position (2) of a fluorescently labeled calmodulin, associated with only one of the necks, was monitored during movement. Here we describe the use of quantum dots (Qdots), which have significant advantages over fluorophores in terms of their brightness, narrow emission spectra, and photostability, so that the heads of a single myosin V molecule were labeled differentially with Qdots having distinct emission spectra, and then imaged with sufficient spatial and temporal resolution so that each head was observed alternating positions in a hand-over-hand fashion during processive movement.

An expressed double-headed heavy meromyosin V with a C-terminal yellow fluorescent protein (YFP-HMM_{M5}) was modified by the addition of a biotin tag to its N-terminus (YFP-$HMM_{M5\text{-}BIO}$) (3). This served as an attachment site for streptavidin-functionalized Qdots with emission spectra centered at 565 or 655 nm (Quantum Dot Corp., Hayward, CA). By incubating a mixture of $Qdot_{565}$ and $Qdot_{655}$ in fourfold excess over YFP-$HMM_{M5\text{-}BIO}$, a solution was obtained containing free Qdots and a mixture of myosin species, one of which had each head labeled with either a $Qdot_{565}$ or a $Qdot_{655}$. The Qdot-myosin mixture was introduced into a 10-μL flow-cell chamber in which actin filaments were adhered to a glass surface through *N*-ethylmaleimide-modified myosin. The final assay buffer was 25 mM KCl-actin buffer and the desired [ATP] at 24°C as in Baker et al. (3). Fluorescence imaging was through an objective-type total internal reflectance microscope, as previously described (3), with a software-controlled filter wheel to switch between the two Qdot emission filters. Qdots were excited with the 514-nm argon laser line. Images were captured at an 83-ms integration time, switching between colors every 250 ms. Typically 300 pairs of images were recorded for a total of 150 s. Digital images were first corrected for image registration error, based on an array of pairs of streptavidin-$Qdot_{565}$ bound to biotin-$Qdot_{655}$. A Qdot image associated with a processive myosin was then fitted to a two-dimensional Gaussian, representing the Qdot point-spread function (2). With this analysis, the x,y-position of the Qdot was determined with 6-nm resolution at the 83-ms integration time. Processive run lengths and run times were measured and velocity calculated as the run length divided by run time (3).

Both the biotin tag and the subsequent attachment of Qdots did not affect velocity (0.3 ± 0.1 μm/s, $n = 52$) or run length (1.3 ± 0.1 μm, $n = 52$) because these values for the YFP-$HMM_{M5\text{-}BIO}$ were no different than the control YFP-HMM_{M5} at 1 mM ATP (3). Experiments were performed at 300 nM ATP resulting in a slow, 0.3 s^{-1}, myosin V stepping rate as the motor paused for significant periods, thus allowing the

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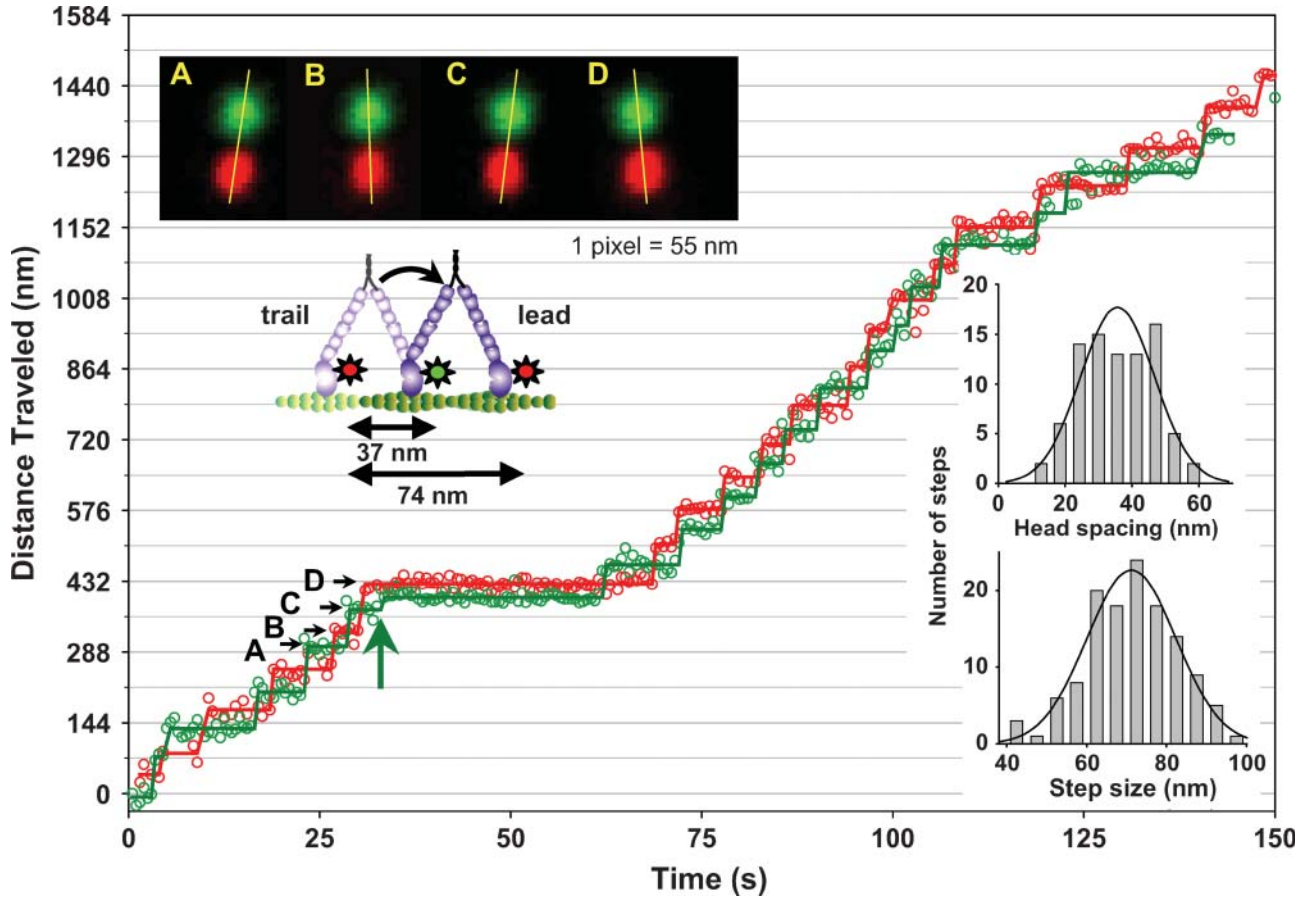

FIGURE 1 Myosin V processive run with heads labeled with different colored Qdots. Green and red open circles are the $Qdot_{565}$ and $Qdot_{655}$ positions, respectively, determined by Gaussian fits. Solid lines are the average Qdot positions between steps with the onset of steps determined by eye. Upper left are averaged Qdot images for steps labeled *A–D*, with red and green images offset by 12 pixels in *y* for clarity. The yellow lines connect Qdot centers emphasizing alternating relative head positions. Green arrow identifies substep. Lower right are histograms of interhead spacing and step size.

position of each myosin V head (i.e., Qdot position) to be defined through multiple images. Under these conditions, individual myosin V molecules were easily observed taking discrete steps based on the movement of individual Qdot images (Fig. 1). To identify a myosin V with its heads differentially labeled with one $Qdot_{565}$, viewed as green, and one $Qdot_{655}$, viewed as red, composite color image movies were generated so that a moving yellow Qdot represented the overlap or colocalization of a green and red Qdot.

If a hand-over-hand mechanism defines myosin V movement as it travels along the top surface of the actin filament due to the experimental setup, then the head positions relative to each other and the displacement taken by each head during a step are constrained by the dimensions of the actin filament helical repeat. Specifically, with both heads attached to actin, the long six-IQ neck allows the myosin V to span the actin repeat (4) so that each head is bound to an equivalent actin monomer ~37 nm apart (prediction No. 1). Secondly, as the trailing head releases and takes a forward step it will move by ~74 nm to bind to the next available equivalent actin binding site (2) (prediction No. 2). Finally, for the mechanism to be hand-over-hand, the heads must alternate positions taking turns being the leading head (prediction No. 3). In Fig. 1, the movement of a differentially labeled myosin V as it takes 19 steps over 150 s is presented. By visual inspection it is apparent that the heads are ~36 nm apart when not moving and then alternate positions after the trailing head takes its ~72-nm step. For all myosin V molecules studied, the heads were 36 ± 11 nm ($n = 97$) apart while paused, followed by the trailing head taking a 72 ± 10 nm ($n = 127$) step to become the leading head (histograms in Fig. 1). These data, through direct observation, strongly support the hand-over-hand model given that all three model predictions were confirmed. Preliminary data with similar results have been recently reported using fluorophore-labeled calmodulins to decorate the neck (5,6).

The use of Qdots in the single myosin V molecular motor assay offers a high-resolution view of the dynamics

of each head as it strides along actin. For example, an occasional substep was observed (Fig. 1, *green arrow*), suggesting that once the head is stably attached to an actin monomer, it can detach and reattach before taking its 72-nm step. Substeps have been observed in the laser trap (7–9), but these are most likely due to conformational changes associated with rotation or bending of the lever-like neck that are transmitted to the myosin V C-terminus (1,4,10) rather than changes in head position. We did not detect any backward steps for the freely moving myosin V molecules described here as compared to those observed in the laser trap where the myosin experiences an externally applied load (7,8,9). However, by using Qdot-labeled myosin V in the laser trap, one can begin to monitor not only the mechanics of the entire molecule but the structural dynamics of each head as the myosin V generates force and motion. Therefore, the Qdot fluorescence characteristics offer the opportunity to label the myosin motor, or any protein undergoing conformational changes, in multiple locations so that a three-dimensional picture of the structural dynamics can be compiled in real time.

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