RESULTS AND DISCUSSION (II)

Single molecule high resolution colocalization (SHREC)

To further expand the potentials of single molecule fluorescence microscopy, a new technique was developed that allowed tracking two fluorophores simultaneously with nanometer resolution in a total internal reflection fluorescence microscopy assay[68]. To circumvent the diffraction limited resolution of about 250 nm for visible light, two chromatographically different fluorophores were used. Myosin V is an ideal molecule to study with this new technology. Numerous elaborate experiments strongly suggest that myosin V utilizes its long lever arms to walk hand-over-hand on actin[16,42,45,46,78]. Once attached to the actin filament, myosin V undergoes multiple ATPase cycles which in turn lead to several hand-over-hand steps before the molecule dissociates. Thus, the relative position of the myosin V heads periodically alternate as the molecule walks processively along actin (Figure 51). The goal was to follow the position of both heads simultaneously with nanometer resolution in real time and directly demonstrate the alternation of the heads as a result of the hand-over-hand walking mechanism.

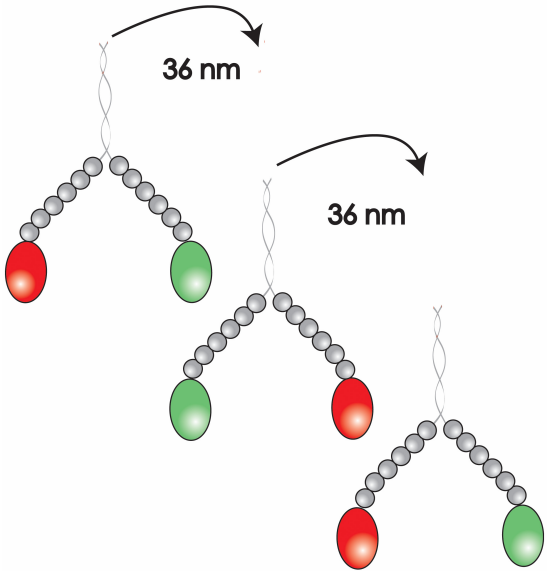

Figure 51: The relative position of the heads alternate while the protein processively walks hand-over-hand on actin. In a single molecule TIR experiment the position of the differentially labeled heads will alternate every 36 nm with each step.

Construct Design and Protein Expression

Myosin V expression plasmids encoded for a myosin C-terminally fused to the YFP gene. The presence of the YFP fluorophore was found to interfere with the Cy3 emission in the TIR microscope. Consequently, the YFP moiety was deleted in the wild type myosin V construct to eliminate the YFP interference. The resulting construct coded for the myosin V heavy chain and a C-terminal GCN4 coiled coil domain to ensure dimerization (Figure 52).

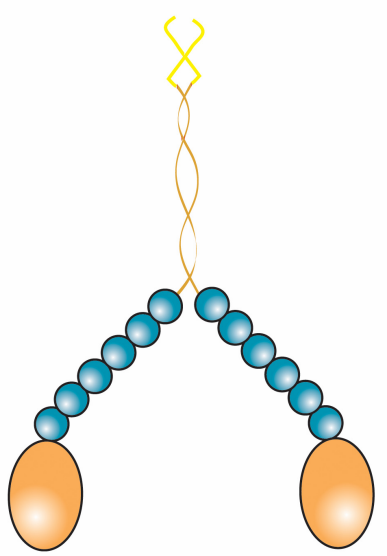

Figure 52: Schematic structure of the YFP-deleted myosin V construct: The wild type head domain and the coiled coil domain that is truncated at Glu-1099 are shown in orange. The yellow end of the coiled coil represents the leucine zipper domain from GCN4. The light chains are shown in blue.

To delete the YFP gene in the p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid primer #1 and primer #2 were designed to PCR part of the heavy chain gene upto a unique restriction site in the myosin V coding sequence. Primer #1 contained a sequence coding for the C-terminal FLAG tag, which was originally C-terminal to the YFP gene. Primer #2 was designed for the unique restriction sites in the middle of the open reading frame (Figure 53).

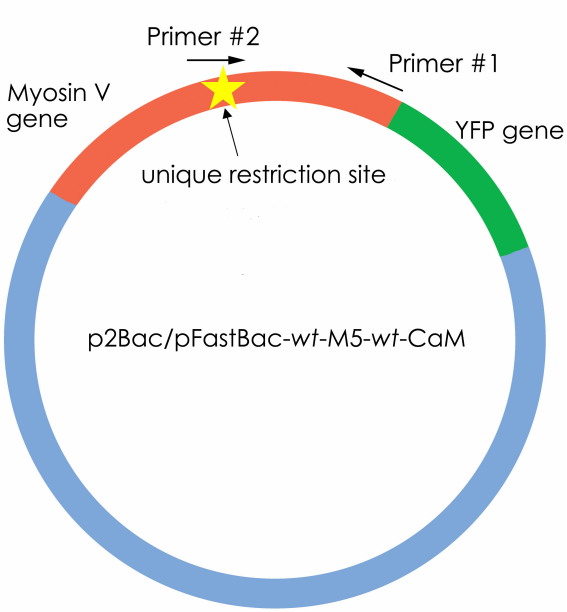

Figure 53: Schematic presentation of the YFP gene deletion strategy in the p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid. Blue represents the backbone of the plasmid. The YFP gene is shown in green and the myosin V gene is shown in red, respectively. Primer #1 that was complementary to the C-terminal myosin V gene was combined with primer #2 to delete the YFP gene.

The combination of the primer #1 with the primer #2 resulted in a PCR product for C-terminal part of the YFP deleted myosin V gene. The PCR product was restriction digested and inserted into the p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid that was restriction digested using the same enzymes. The resulting p2Bac/pFastBac-Δ*YFP*-M5-*wt*-CaM plasmid coded for the YFP deleted myosin V and the wild type calmodulin gene. The coding sequence was DNA sequenced to confirm the YFP deletion and to ensure that no secondary mutations were introduced.

Expression and Purification of the YFP-deleted Myosin V

The p2Bac/pFastBac-Δ*YFP*-M5-*wt*-CaM plasmid was transposed into the bacmid plasmid and the Sf9 cells were infected with the bacmid plasmids to amplify virus for myosin V protein expression as described previously for the myosin VI construct. The myosin V protein was purified via batch binding to the anti-FLAG resin as described previously.

Direct Demonstration of Hand-over-Hand Walking of Myosin V

To directly observe the alteration of the myosin V catalytic heads during processive stepping, the molecule was dually labeled with Cy3 and Cy5 dyes on its calmodulin light chains. To this end, Cy3 and Cy5 dye labeled calmodulins were exchanged onto the myosin V protein *in vitro* by increasing the calcium ion concentration. Preferentially, the exchange reaction occurs with calmodulins next to the head[45]. Thus, following the dye labeled calmodulins would consequently report on the position of the catalytic head domains. The fluorophores were imaged separately and localized individually with nanometer precision (Figure 54). The registration between the two imaging channels was measured by using fiduciary markers, and the centers of the two probes were mapped onto the same space.

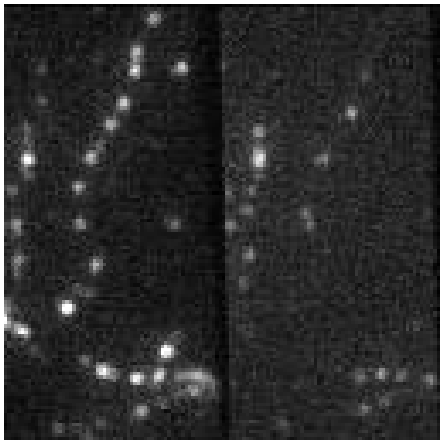

Figure 54: The Cy3 dye is imaged in the left channel and Cy5 dye is imaged in the right channel, respectively.

The dually labeled myosin V heads with two different dyes were shown to alternate while the motor walked processively along actin (Figure 55).

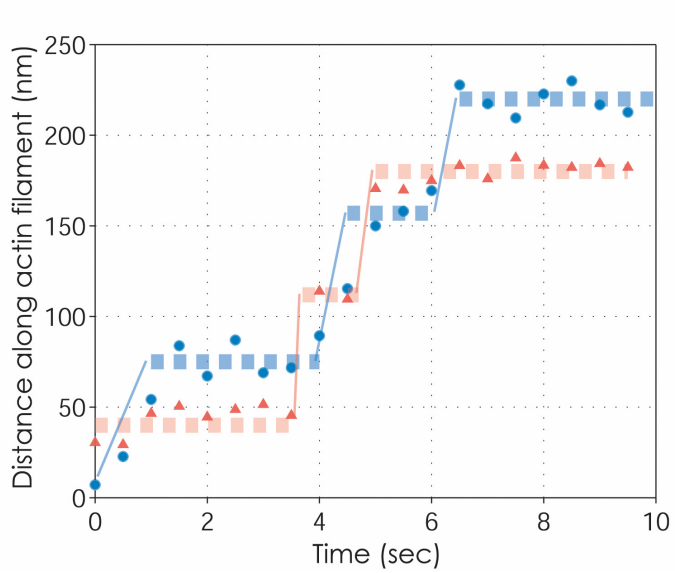

Figure 55: A single myosin V molecule walking on an actin filament. The heads swap orientation as the motor walks.

SHREC allowed the measurement of interfluorophore distances with >10 nm resolution. The capability of probing interactions on the distance scale of 10 nm – 200 nm is critical for understanding the biological macromolecules and their mechanisms of action. Single molecule fluorescence techniques such as single molecule FRET (smFRET) for example provide invaluable insights into the interactions of macromolecules on a distance scale up to 10 nm. With a lower resolution limit of ~10 nm, SHREC can span the gap between the upper resolution limit of smFRET (~10 nm) techniques and the lower resolution limit of far field fluorescence microscopy (~250 nm).