Materials and Methods (II)

Single Molecule High Resolution Colocalization (SHREC)

A novel single molecule high resolution dual color colocalization TIR microscopy technique was developed that allows interfluorophore distance measurements with nanometer precision[68]. The technique was successfully applied to directly demonstrate the hand-over-hand walking mechanism of myosin V by measuring the interfluorophore distances between two differentially labeled catalytic heads during processive stepping[68]. The C-terminal YFP gene in the myosin V construct was deleted in the p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid to eliminate the interference of the YFP emission in the Cy3 dye detection channel. To dually label the resulting myosin V construct, the calmodulin light chains were exchanged with sub-stoichiometric amounts of Cy3 and Cy5 labeled calmodulin *in vitro*.

YFP Deletion in the p2Bac/pFastBac-*wt*-M5-*wt*-CaM Plasmid

The p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid was used as a template to delete the C-terminal YFP residue in the myosin V construct. Sph I and Spe I restriction sides were introduced into the primers to facilitate cloning. The forward primer was complementary to the Sph I unique restriction site in the myosin V heavy chain. The complimentary sequence of the reverse primer started at C-terminal end of the GCN4 residue to delete the YFP gene (Figure 53).

Forward Primer (Fwd 06)

5'- ATAGCATGCGAGATGCTACTATTGCTC -3'

Reverse Primer (Rev 06)

5'- TATACTAGTTCACTCCCCGACAAGCTTCTTAAGT -3'

PCR Reaction

100 ng Template DNA

200 nM Forward primer

200 nM Reverse primer

1 µl (10 mM) dNTP mix

2 µl (50 mM) $MgSO_4$

5 µl (10X) Reaction buffer

0.2 µl (5U/µl) Platinum® *Taq* DNA Polymerase HiFi

50 µl Final volume

PCR Method

Step	Time	Temperature	Cycles
Initial Denaturation	1 min	94°C	1X
Denaturation	30 s	94°C	30X
Annealing	30 s	55°C	
Extension	5 min	68°C	
Final Extension	5 min	72°C	1X

Restriction digest reaction of the PCR product

15 µl PCR product

1 µl Sph I (5000 U/ml)

1 µl Spe I (10000 U/ml)

1 µl BSA (10 mg/ml)

2 µl (10X) NEB 2 buffer

20 µl Final volume

Restriction digest reaction of the p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid

3 µl p2Bac/pFastBac-*wt*-M5-*wt*-CaM plasmid (Miniprep)

1 µl Sph I (5000 U/ml)

1 µl Spe I (10000 U/ml)

1 µl BSA (10 mg/ml)

2 µl (10X) NEB 2 buffer

10 µl final volume

Ligase reaction to create the p2Bac/pFastBac-Δ*YFP*-M5-*wt*-CaM plasmid

Uncut Vector Control	Vector Religation Control	Ligase Reaction	
1.5 µl	1.5 µl	1.5 µl	10X Ligase Buffer (NEB)
0 µl	0 µl	10.5 µl	Spe I / Sph I digested PCR product
1 µl	1 µl	1 µl	Spe I / Sph I digested p2Bac/pFastBac-*wt*-M5-CaM^{m} plasmid
0 µl	1 µl	1 µl	T4 DNA Ligase (1U / µl) (NEB)
12.5 µl	11.5 µl	1.5 µl	10 mM Tris pH 8.5

The ligase reactions were transformed into *E. coli* DH5-α cells and plated on LB plates containing (0.1 mg/ml) ampicillin as described previously. Single colonies were picked and grown over night in 6 ml LB media complemented with (0.1 mg/ml) ampicillin. The plasmid DNA was isolated via QIAprep® Spin Miniprep Kit as previously described. The YFP deletion was confirmed via restriction digest analysis and custom DNA sequencing.

Restriction digest analysis of the p2Bac/pFastBac-Δ*YFP*-M5-*wt*-CaM plasmid

1 µl Plasmid DNA

1 µl Sph I (5000 U/ml)

1 µl Spe I (10000 U/ml)

1 µl BSA (10 mg/ml)

1 µl (10X) NEB 2 buffer

10 µl Final volume

Viral Amplification and Protein Expression of the YPF deleted Myosin V construct

The transposition and viral amplification of the p2Bac/pFastBac-Δ*YFP*-M5-*wt*-CaM plasmid, as well as the protein expression and purification were carried out as described in the previous chapter.

Cy3 and Cy5 Labeled Calmodulin Exchange onto the YFP-deleted Myosin V Construct

To directly observe the alternation of the myosin V catalytic heads while the protein processively walked hand-over-hand along actin filaments in a single molecule TIRF microscope, the calmodulins on the wild type myosin V protein were exchanged with Cy3 and Cy5 dye labeled calmodulin proteins *in vitro* by increasing the calcium ion concentrations.

0.15 μM myosin V, 0.22 μM wild type calmodulin, 0.55 μM Cy3 labeled calmodulin and 0.73 μM Cy5 labeled calmodulin in 50 μl exchange buffer were incubated for 2 minutes at room temperature. The calcium concentration was raised to 1 mM to initiate the exchange reaction. After 5 minutes incubation at room temperature, the reaction was quenched with 8 mM EGTA. The exchange reaction was applied to a Nanosep 100K Omega spin column (Pal Corporation) and centrifuged at 8000 rpm for 1 minute in a table top centrifuge. The volume of the flow through was measured and the remaining volume in the spin column was brought to 100 μl by adding the measured volume difference using AB buffer. This centrifugation step was repeated three times. In the last two centrifugation steps the volume in the spin column was brought to 100 μl using AB buffer complemented with 90 nM unlabeled wild type calmodulin. After the last spin the volume of the FT was measured to determine the remaining volume in the spin column. The remaining volume in the spin column was added to 50 μl final volume using AB buffer with 90 nM unlabeled wild type calmodulin. To recover the protein the bottom of the spin column was flicked very carefully and the protein was pipetted out. The labeled myosin V was kept on ice and used the same day of the experiment.

Single Molecule In vitro Motility Assays

The microscope set up and the single molecule in vitro motility assays were performed as described in the previous chapter except for the flow cell preparation. No special actin suspension methods were required as myosin V

can walk processively on entirely surface attached actin filaments via biotin-avidin interactions.

Flow Cell Preparation

The flow cell geometry was essentially the same as in the single molecule studies of myosin VI (Figure 23). Unlabeled phalloidin actin was used in this assay because the actin detection channel was used for Cy3 dye detection simultaneously with the Cy5 dye detection. The order of addition was as follows:

1. 15 µl (1 mg/ml) Biotin-BSA
2. 30 µl Assay buffer-BSA
3. 15 µl (0.5 mg/ml) NeutrAvidin
4. 30 µl Assay buffer-BSA
5. 15 µl (250 nM) Biotinylated phalloidin actin
6. 100 µl Assay buffer-BSA
7. 20 µl Motility buffer

Two minutes of incubation was allowed after each addition except after step 5 which was 5 minutes.

Assay Buffer-BSA

25 mM KCl

25 mM Imidazole, pH 7.5

1 mM EGTA

4 mM $MgCl_2$

10 mM DTT

1 mg/ml BSA

Motility Buffer

10 mM Tris, pH 8.0

50 mM NaCl

10 mM $MgCl_2$

17 U Glucose oxidase

260 U Catalase

0.4% (v/v) Glucose

1% β-Mercaptoethanol (v/v)

300 nM ATP

1 mM Creatine phosphate

0.1 mg/ml Creatine phosphokinase

0.5% (v/v) Triton-X 100

5 μM wild type CaM

CaM exchanged myosin V