

Material and Methods (I)

Dissection of the Myosin VI Walking Mechanism

To investigate the myosin VI walking mechanism in a single molecule TIR experiment the wild type myosin VI protein was tagged with a reactive mutant cysteine or with a specific mutant cysteine motif on its otherwise cysteine free calmodulin light chain. The presence of a reactive cysteine (KKCK-tag) or a specific cysteine motif (CCRECC-tag) allowed specific fluorescent labeling of the introduced mutant cysteines in the presence of the wild type cysteines in the myosin VI protein.

Overview of the Cloning Strategy of KKCK- and CCRECC-tagged Calmodulin Gene

The wild type calmodulin (CaM) gene in the p2Bac/pFastBac-wt-CaM plasmid was PCR amplified using mutagenesis primers that contained the in-frame fusions of the N-terminal KKCK- and CCRECC-tags, respectively. To facilitate cloning, Not I and Sal I restriction sites were introduced at the ends of the primers. The PCR amplified mutant calmodulin genes (CaM^m) containing the N-terminal KKCK- or CCRECC-tags were cloned into the Not I and Sal I digested p2Bac/pFastBac-wt-CaM plasmid to give p2Bac/pFastBac-CaM^m. The introduced mutations were confirmed via custom DNA sequencing.

To create the p2Bac/pFastBac-wt-M6-CaM^m plasmid, the EcoR I and BamH I digested myosin VI gene was cloned into the EcoR I and BamH I digested p2Bac/pfastBac-CaM^m plasmid containing the N-terminally KKCK- or CCRECC-tagged calmodulin gene. The resulting plasmid contained the mutagenized CaM gene under the p10 promotor and the wild type myosin VI gene under the polyHedrin promotor, respectively (Figure 16). The myosin VI gene on the p2Bac/pFastBac plasmid codes for porcine myosin VI that was truncated at Ala-990 to create a double headed myosin VI construct including 20 wild type heptad repeats of the predicted coiled-coil. To ensure dimerization, a leucine zipper (GCN4) was added at the C-terminal end of the wild type coiled-coil.

The leucine zipper was followed by YFP (S65G, S72A, K79R, T203Y, common name *Topaz*⁷⁰) and a FLAG[®] tag (GDYKDDDDK) to facilitate purification.

Standard Molecular Biological Methods

DNA Analysis Methods

Agarose Gel Electrophoresis

DNA fragments were separated via (1%) agarose gel electrophoresis. The gels were prepared by dissolving the agarose in the TAE buffer. To visualize the DNA, 5 µl of (10 mg/ml) ethidium bromide was added to the 60 ml (1%) agarose solution. To load the samples, the DNA was mixed in equal volume ratios with the agarose gel loading buffer. Electrophoresis was performed at 100 V. The DNA was detected using UV light and the size of the DNA was determined using standard 1Kb DNA ladder.

1L (50X) TAE Buffer

242 g Tris-base
57 ml (glacial) Acetic acid
100 ml (0.5 M) EDTA, pH 8.0

Agarose Gel Loading Buffer

20% Glycerol
0.1 M EDTA
1% SDS
0.25% Bromophenol blue

DNA Purification and Isolation Protocols

DNA Isolation via QIAprep[®] Spin Miniprep Kit

To isolate plasmid DNA, single colonies selected from the *E. coli* DH5- α transformation were grown at 37°C to saturation over night in 6 ml LB media complemented with 0.1 mg/ml ampicillin. The cells were removed into sterile (15 ml) Falcon tubes and centrifuged in a J-6M swinging bucket centrifuge (Beckman) for 15 minutes at 4000 rpm. The pelleted cells were resuspended in

250 µl of the Qiagen P1 buffer and transferred into 1.5 ml sterile Eppendorf tubes. To lyse the cells, 250 µl of the Qiagen P2 buffer was added to each tube and the tubes were carefully inverted six times before neutralizing with 350 µl of the Qiagen N3 buffer. The mixture was centrifuged in a benchtop centrifuge at 14000 rpm for 10 minutes. The supernatant was removed and applied onto the QIAprep® spin column. The columns were centrifuged in a benchtop centrifuge at 14000 rpm for 1 minute and washed with 750 µl of the Qiagen PE buffer by centrifuging at 14000 rpm for 1 minute. The wash buffer was discarded and the spin was repeated to quantitatively remove the PE buffer. The DNA was eluted by applying 40 µl of 10 mM Tris buffer (pH 8) onto the column and incubating for 1 minute and spinning at 14000 rpm for 1 min. The DNA was stored at -20°C.

Agarose Gel Purification of DNA via QIAquick® Gel Extraction Kit

All PCR products and restriction digest reactions were purified from agarose gel prior further use. The DNA bands on the agarose gels were visualized using a non-mutagenic UV lamp (365 nm). The DNA containing piece of agarose was transferred into a sterile 1.5 ml Eppendorf tube. To dissolve the agarose gel, 500 µl of the Qiagen QG buffer was added and the tube was incubated in a 37°C water bath for 20 minutes or until the agarose gel was completely dissolved. The solution was applied onto a QIAquick® spin column and centrifuged in a benchtop centrifuge for 1 min at 14000 rpm. 750 µl of the Qiagen PE buffer was applied onto the column and centrifuged for 1 min at 14000 rpm. The wash buffer was discarded and the spin was repeated to quantitatively remove the PE buffer. The DNA was eluted by applying 40 µl of 10 mM Tris buffer (pH 8) onto the column and spinning at 14000 rpm for 1 min after an incubation time of 1 min.

Restriction Digest Reactions of DNA

All DNA restriction digest reactions using restriction endonucleases were carried out under optimized conditions as described by the providers.

The analytical restriction digest reactions were incubated for one hour and the preparative restriction reactions were incubated for 3 hours, respectively. The products of the preparative restriction digest reactions were purified from agarose gel using QIAquick® Gel Extraction Kit prior to the DNA ligase reactions.

Transformation of Chemically Competent *E. coli* DH5- α Cells

The chemically competent *E. coli* DH5- α cells were thawed on ice and 50 μ l aliquots were incubated with 1-10 ng DNA for each transformation on ice for 30 minutes. The heat shock was performed at 42°C in a water bath for 45 seconds. After the heat shock the tubes were transferred immediately onto ice and incubated for an additional 2 minutes before they were plated on an LB plate supplemented with (0.1 mg/ml) ampicillin. The plates were incubated over night at 37°C to grow single colonies.

Protein Analysis Methods

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein bands were separated using SDS-PAGE under denaturing conditions. Depending on the size of the investigated protein 10% or 15% polyacrylamide gels were used. To analyze the calmodulin light chain protein in a 15% polyacrylamide gel, 2 mM EGTA was added into the stacking and separating gel buffers to remove calcium ions. The protein samples were boiled for 5 minutes at 100°C in 1X SDS sample buffer before loading. The gels were run in 1X electrophoresis buffer at 20 - 40 mA with 250 V limit for 45 - 60 minutes. Protein bands were stained with Coomassie Brilliant Blue by constant shaking at 20 - 60 rpm for one hour. The polyacrylamide gel was destained for one hour by constant shaking at 20-60 rpm. The molecular weight of the protein bands were determined using standard low-range or high-range protein markers.

Stacking Gel Buffer (sterile filtered)

140 mM Tris (pH 6.8)

0.1% SDS

Separating Gel Buffer (sterile filtered)

560 mM Tris (pH 8.8)

0.1% SDS

5X SDS Protein Sample Buffer

250 mM Tris (pH 6.8)

500 mM DTT
10% (w/v) SDS
0.5% (w/v) Bromophenol Blue
50% (v/v) Glycerol
stored at -20°C

Coomassie Brilliant Blue Stain

0.25% (w/v) Coomassie Brilliant Blue R 250
40 % Methanol
10% Acetic acid

Destain Buffer

40% Methanol
10% Acetic acid

2L (10X) Electrophoresis Buffer

60.55 g Tris Base (pH 8.3)
288.4 g Glycine
20 g SDS

Determination of the Protein Concentration

Spectroscopic Determination

The C-terminal YFP moiety in myosin VI construct was exploited to determine the protein concentration. The concentration was calculated as follows:

$$\text{Absorption (YFP}_{514\text{ nm}}) = c \cdot d \cdot \epsilon$$

$$\epsilon_{\text{YFP}} = 95400 \text{ M}^{-1}\text{cm}^{-1}, 70$$

$$d = 1\text{ cm}$$

Colorimetric Determination

Bradford assay exploits the fact that the Brilliant Blue (G250) dye binding to a protein in an acidic solution leads to differential changes in the color depending on the protein concentration. The absorbance maximum of the acidic solution shifts from 465 nm to 595 nm when bound to the protein. The

optimized protocol by the provider was used to determine the protein concentration. The C-terminally YFP tagged myosin VI was used as a standard to determine the concentration of the YFP-deleted myosin VI and myosin V constructs, respectively.

Protein Expression in Baculovirus Expression System

Advantages of the Baculovirus Protein Expression System

The baculovirus system is ideal for eukaryotic hetero-oligomeric protein expression because it allows co-infection of cells with two or more viruses that contain the respective heterologous genes. As with other eukaryotic protein expression systems, the baculovirus system allows post-translational modification, correct folding and oligomerization of recombinant proteins. Consequently, the over-expressed protein exhibits proper biological activity and function. A significant advantage of baculovirus expression systems is the high yields in protein expression.

Baculoviruses infect primarily insects with a narrow host range, namely two lepidopteran (moth) families in nature. Thus, working with baculoviruses is considered safe for humans. *Autographa californica* nuclear polyhedrosis virus is the most commonly used baculovirus for protein expression in the insect cells *Spodoptera frugiperda*. The cytoplasmic environment in insect cells permits proper folding and disulfide bond formation, which is not possible in *E. coli* due to the reducing environment in their cytoplasm.

The unconventional myosin V and myosin VI expressed in *E. coli* is not functional due to the lack of required folding machinery and post-translational modifications in the host cells. Thus, the Bac-To-Bac® baculo-virus expression system was used for the expression of unconventional myosins and their mutants. The Bac-To-Bac® method makes use of a dual vector meaning that two genes can be expressed from the same plasmid using two separate promoters (Figure 25). In this case, complicated co-infections become

unnecessary. All specifications in the protocol refer to the myosin V and myosin VI protein expression only. Protocol may differ significantly for other proteins.

Protein Expression in the Baculovirus Expression System

The unconventional myosins, myosin VI and myosin V described in this work were expressed in the Bac-To-Bac® baculovirus expression system. A flow-scheme illustrates the main steps of the protein expression (Figure 15).

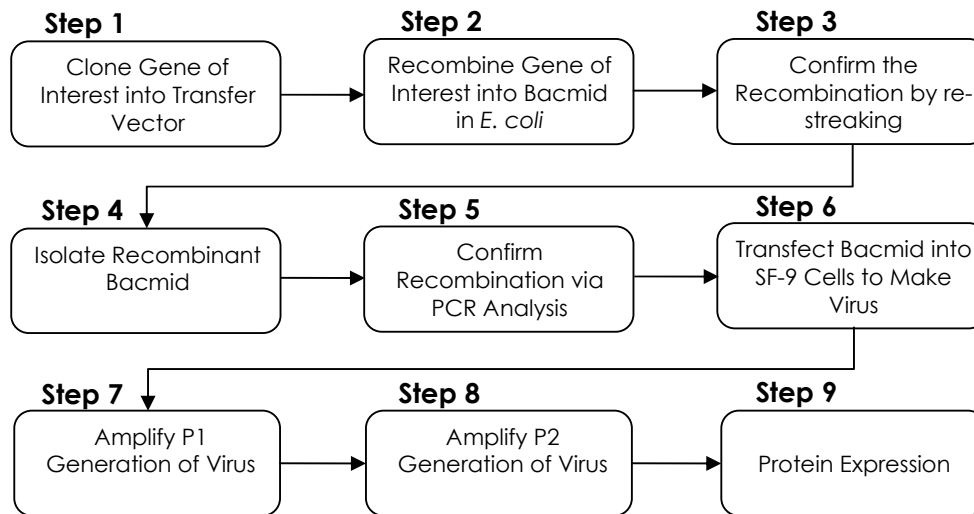


Figure 15: Overview of the steps involved in the Bac-To-Bac® baculovirus protein expression system.

Construction of the Recombinant Transfer Vector for Protein Expression -Step 1

A hybrid transfer vector consisting of the commercially available p2Bac and pFastBac vectors (Invitrogen) was used to express myosin VI and myosin V in insect cells (Figure 16). The combination of two plasmids with two strong baculovirus promoters allows simultaneous expression of two different proteins. Furthermore, the transfer plasmid contains the transposition sites that are necessary for the recombination into the bacmid (Step 2).

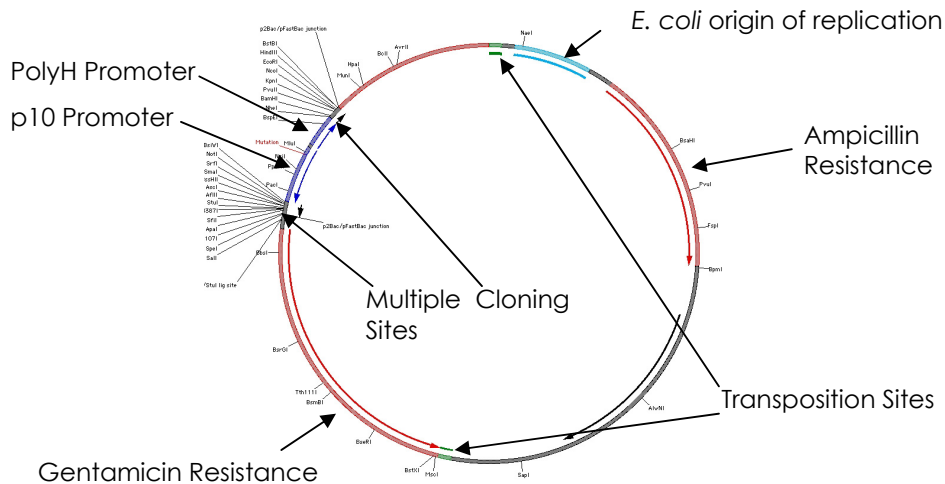


Figure 16: Map of the p2Bac/pfastBac donor vector. The multiple cloning sites following the PolyH and p10 promoters allow the simultaneous insertion of the myosin heavy chain gene and the corresponding light chain gene into one vector.

Generation of the Recombinant Bacmid -Step 2 and Step3

The recombination of the transfer vector with the baculovirus shuttle vector (bacmid) is achieved by a site specific transposition (Figure 17). The bacmid is then propagated in DH10Bac™ *E. coli* cells. DH10Bac™ competent cells contain the bacmid with a mini-attTn7 target site and the helper plasmid. The mini-Tn7 element on the p2Bac/pFastBac donor plasmid can transpose to the mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Successful recombination is identified by the disruption of the lacZ gene on the bacmid, allowing a blue/white screening in the presence of Bluo-Gal and IPTG. The white colonies are re-streaked to confirm phenotype.

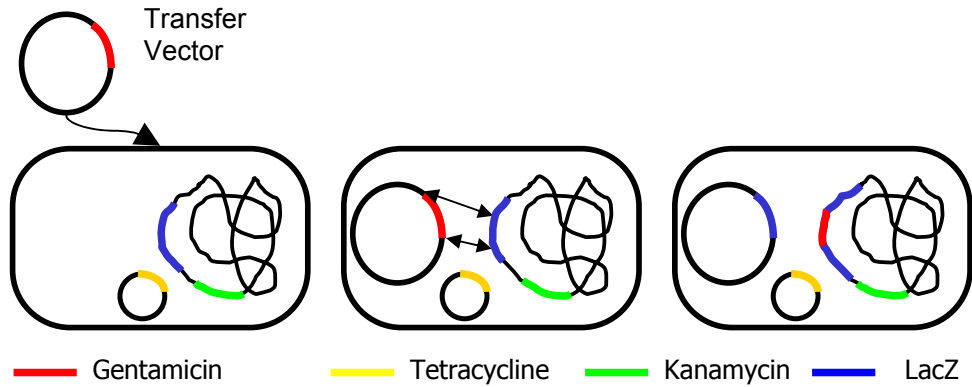


Figure 17: Generation of recombinant baculovirus.

Isolation of the Recombinant Bacmid and Conformation of Recombination via PCR -Step 4 and Step 5

The colonies are grown over night in LB supplemented with the antibiotics and the recombinant bacmid is isolated. The insertion of the gene of interest is confirmed by PCR amplification using pUC/M13 primers (blue bars) sites built into the vector on either side of the recombination site. As it was the case in this work, too large insertions (shown in red) may require internal primers illustrated as green bars in Figure 18.

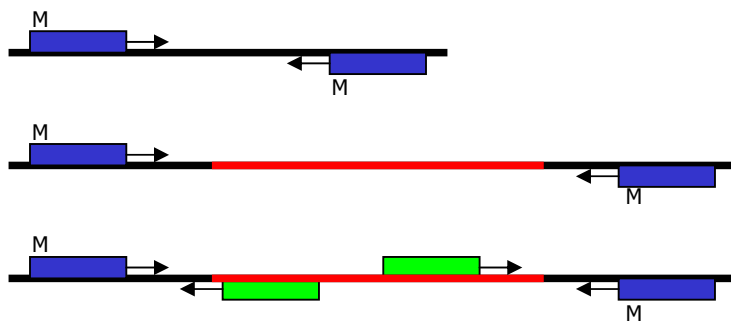


Figure 18: PCR-confirmation of the recombination using external and internal primers.

Transfection of the SF-9 Cells with the Bacmid -Step 6

The recombinant bacmid is mixed with positively charged lipid Cellfectin® reagent which coats the DNA to facilitate transfection (Figure 19). The

Cellfectin® reagent is a (1:1.5) (M/M) liposome mixture of the cationic lipid N,N',N'',N'''-tetramethyl-N,N',N'',N'''-tetrapalmitylspermine and dioleoylphosphatidylethanolamine in membrane filtered water. The lipid coated bacmid enters the Sf-9 cells after 5 hours incubation time at 27°C.

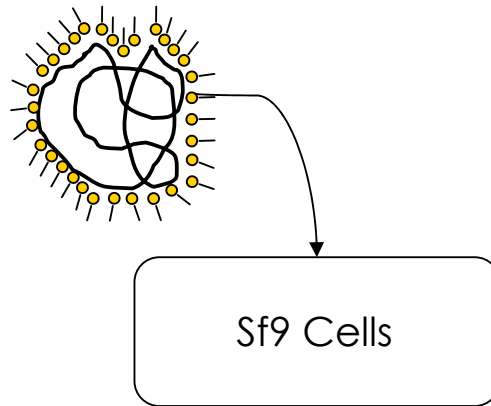


Figure 19: The lipid coated bacmid. The positively charged lipid Cellfectin® (shown in yellow) facilitates the transfection of the Sf9 cells.

After 72 hours the amplified virus is harvested from the media (Figure 20). The first virus generation is called the P₀ virus.

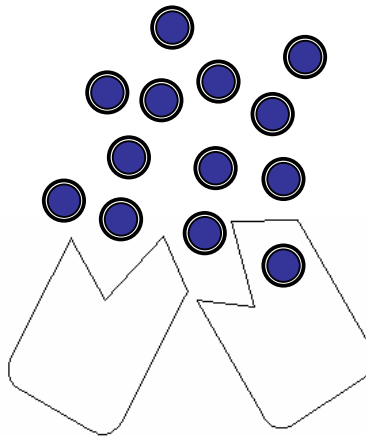


Figure 20: After the infection, the virus starts amplifying in the Sf9 cell and eventually leads to lysis. The free virus can then infect other Sf9 cells.

Amplification of the P₁ and P₂ Generations – Step 7 and Step 8

For the virus amplification, >97% viable Sf9 cells are infected at a density of 5×10^5 cells/ml. For the P₁ viral amplification a volume ratio of 100 μ l P₀ virus (1.8×10^8 pfu/ml) to 100 ml Sf9 cells (5×10^5 cells/ml) is required. For the P₂ viral amplification a volume ratio of 250 μ l P₁ virus (1.8×10^8 pfu/ml) to 1000 ml Sf9 cells

(5×10^5 cells/ml) is required. The amplified virus is harvested after the viability of the Sf9 cells dropped under 10%. The viral amplifications usually take one week. The harvested virus is stable and can be stored light protected at 4°C for several months. Before starting the protein expression the titre for the P₂ amplification is determined as described in Materials and Methods (I).

Protein Expression – Step 9

For the protein expression, >97% viable Sf9 cells are infected at a density of 2×10^6 cells/ml. The P₂ virus and the Sf9 cells are mixed in a volume ratio of 75 ml of the P₂ (1.8×10^8 pfu/ml) virus and 925 ml Sf9 cells. The protein expression takes about 48 hours.

PCR Mutagenesis and DNA Sequencing Primers

Mutagenesis Primers for N-terminal Insertion of the KKCK- and CCRECC-tags into the Calmodulin Gene

To create a myosin VI construct with an N-terminally **KKCK**-tagged calmodulin light chain, the calmodulin (CaM) gene under the p10 promotor was PCR-amplified from the p2Bac/pfastBac-CaM plasmid (template) using mutagenesis primers that contained the desired mutation. To facilitate cloning, the forward primer contained a **Not I** restriction site and the reverse primer contained a **Sal I** restriction site, respectively. All DNA primers were HPLC purified.

Forward Primer (Fwd 01)

5' ATAG**C**CGG**C**CGCATG**AAGAAGTGTAA**GCAGATCAACTGACAGAAGAGC-3'

Reverse Primer (Rev 01)

5'-GCG**T**CG**A**CTCACTTCGCTGTCATCATCTG-3'

To create a myosin VI construct with an N-terminally **WEAAAREACC**RECCARA-tagged (short **CCRECC**-tagged) calmodulin light chain, the CaM gene under the p10 promotor was PCR-amplified from the p2Bac/pfastBac-CaM plasmid (template) using mutagenesis primers that contained the desired mutation. To

facilitate cloning, the forward primer contained a **Not I** and the reverse primer contained a **Sal I** restriction site, respectively.

Forward Primer (Fwd 02)

5'-ATA**GCGGCCGC**ATGTGGGAGGCCGCTGCTAGGGAGGCCTGC
TGCAGGGAGTGCTGCGCTAGGGCCGCAGATCAACTGAC-3'

Reverse Primer (Rev 02)

5'-G**CGTCGACT**CACTTCGCTGTCATCATCTG-3'

DNA Sequencing Primers for Confirmation of the KKCK- and CCRECC Insertions

The introduced mutations in the p2Bac/pFastBac-wt-CaM^m plasmid were confirmed via DNA sequencing using the following primers:

Forward Primer (Fwd 03)

5'-GCCGGGACCTTTAATTCAACCCAACAC-3'

Reverse Primer (Rev 03)

5'-AAATTGCGGCCGCCATGGCAGATCAACTGACAGAAGAGC-3'

Primers for PCR Analysis of the Recombinant Bacmid

The transposed bacmids were analyzed in two different PCR reactions using primer pairs Fwd 04/Rev04 and Fwd 04/Rev04, respectively.

M13 Forward Primer (Fwd 04)

5'-GTTTCCCAGTCACGAC-3'

GenR#1 Reverse Primer (Rev 04)

5'-ATCATCGCGCTTGCTGCC-3'

pGFP#4 Forward Primer (Fwd 05)

5'-CTTTCGGGCATGGCGGAC-3'

M13 Reverse primer (Rev 05)

5'-CAGGAAACAGCTATGAC-3'

PCR Reactions

Insertion of the KKCK- and CCRECC-Tags into the p2Bac/pFastBac-wt-CaM Plasmid

100 ng p2Bac/pfastBac-wt-CaM plasmid (Miniprep)

200 ng Forward primer

200 ng Reverse primer

200 μ M dNTP mix

5 μ l (10X) Reaction buffer

1 μ l (2.5 U/ μ l) Pfu Turbo DNA polymerase (Invitrogen)

50 μ l Final volume

PCR Method

Step	Time	Temperature	Cycles
Initial Denaturation	5 min	95°C	1X
Denaturation	30 s	94°C	25X
Annealing	30 s	54°C	
Extension	1 min	72°C	
Final Extension	5 min	72°C	1X

PCR Analysis of the Recombinant Bacmid Plasmid

100 ng Bacmid DNA

1.25 μ l (10 μ M) Forward primer

1.25 μ l (10 μ M) Reverse primer

1 μ l (10 mM) dNTP mix

1.5 μ l (50 mM) MgCl₂

5 μ l (10X) Reaction buffer

0.5 μ l (2.5 U/ μ l) Taq DNA Polymerase (Invitrogen)

38.5 μ l ddH₂O

50 μ l Final volume

PCR Method

Step	Time	Temperature	Cycles
Initial Denaturation	3 min	93°C	1X
Denaturation	45 s	94°C	25X
Annealing	45 s	55°C	
Extension	5 min	72°C	
Final Extension	7 min	72°C	1X

Restriction Digest Reactions

Analytical Restriction Digest Reactions

Confirmation of the KKCK or CCRECC-tagged CaM Gene Insertion

1 µl p2Bac/pfastBac-CaM^m plasmid (Miniprep)

1 µl Sal I (20000 U/ml)

1 µl Not I (10000 U/ml)

1 µl BSA (10 mg/ml)

1 µl (10X) Sal I reaction buffer

10 µl Final volume

Confirmation of the wild type Myosin VI Heavy Chain Gene Insertion

1 µl p2Bac/pfastBac-wt-M6-CaM^m plasmid (Miniprep)

1 µl BamH I (20000U/ml)

1 µl EcoR I (20000 U/ml)

1 µl BSA (10 mg/ml)

1 µl (10X) EcoR I reaction buffer

10 µl Final volume

Preparative Restriction Digest Reactions

Restriction Digest of the PCR Products

15 µl Agarosegel purified PCR product

1 µl Sal I (20000 U/ml)

1 µl Not I (10000 U/ml)

2 μ l BSA (10 mg/ml)
2 μ l (10X) Sal I reaction buffer
21 μ l Final volume

Restriction of the p2Bac/pFastBac-wt-CaM Plasmid

3 μ l p2Bac/pFastBac-wt-CaM plasmid (Miniprep)
1 μ l Sal I (20000 U/ml)
1 μ l Not I (10000 U/ml)
2 μ l BSA (10 mg/ml)
1 μ l (10X) Sal I reaction buffer
10 μ l Final volume

Restriction of the p2Bac/pFastBac- wt-M6-wt-CaM Plasmid

3 μ l p2Bac/pFastBac-wt-M6-wt-CaM plasmid (Miniprep)
1 μ l EcoR I (20000 U/ml)
1 μ l BamH I (20000 U/ml)
2 μ l BSA (10 mg/ml)
1 μ l (10X) EcoR I reaction buffer
10 μ l Final volume

Ligase Reactions

All DNA ligase reactions were carried out under optimized conditions as described by the providers. The reactions were incubated at 25°C for one hour. To ensure a successful ligation the ratio of (9:1) insert to plasmid was maintained.

Ligase Reaction to create the p2Bac/pFastBac-CaM^m plasmid

Uncut Vector Control	Vector Religation Control	Ligase Reaction	
3 μ l	3 μ l	3 μ l	5X Ligase Buffer (Invitrogen)
0 μ l	0 μ l	10.5 μ l	Not I/Sal I digested Mutant CaM ^m gene insert
1 μ l	1 μ l	1 μ l	Not I/Sal I digested p2Bac/pFastBac-wt-CaM plasmid
0 μ l	1 μ l	1 μ l	T4 DNA Ligase (1U/ μ l) (Invitrogen)
11 μ l	10 μ l	0 μ l	ddH ₂ O

Ligase reaction to create the p2Bac/pFastBac-M6-CaM^m plasmid

Uncut Vector Control	Vector Religation Control	Ligase Reaction	
3 μ l	3 μ l	3 μ l	5X Ligase Buffer (Invitrogen)
0 μ l	0 μ l	10.5 μ l	EcoR I/BamH I digested wild type myosin VI gene insert
1 μ l	1 μ l	1 μ l	EcoR I/BamH I digested p2Bac/pFastBac-CaM ^m plasmid
0 μ l	1 μ l	1 μ l	T4 DNA Ligase (1 U/ μ l) (Invitrogen)
11 μ l	10 μ l	0 μ l	ddH ₂ O

Transposition Protocol

Creation of the Bacmid Plasmid in DH10BacTM Cells

The chemically competent *E. coli* DH10BacTM cells were thawed on ice. For each transformation 100 μ l of cells were removed into a sterile 15 ml Falcon tube. The plasmid p2Bac/pFastBac-wt-M6-KKCK-CaM^m or p2Bac/pFastBac-wt-M6-CCRECC-CaM^m (1 ng) was added to the competent cells in 5 μ l final

volume and gently mixed. The mixture was incubated on ice for 30 minutes. After the heat shock for 45 seconds in a 42°C water bath, the Falcon tubes were put on ice for two more minutes. 900 µl S.O.C media was added to each transposition reaction and the tubes were placed in a shaking (225 rpm) incubator at 37°C for 4 hours. The cells were diluted to 10⁻¹, 10⁻², 10⁻³ using S.O.C media. 100 µl of each dilution was plated on the LB-plates supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Blue-Gal, 40 µg/ml IPTG. After 24 hours colonies became visible but for a reliable blue/white screening the plates were grown for 48 hours. To confirm the phenotype, white colonies were re-streaked on supplemented LB-plates and the white colonies were grown in supplemented LB media over night at 37°C.

1 L S.O.C media pH 7.0

20 g Bacto-tryptone

5 g Bacto-yeast extract

0.5 g NaCl

250 mM KCl

10 mM MgCl₂

20 mM Glucose

Isolation of Recombinant Bacmid DNA

Single colonies harvested from the DH10Bac™ transposition were grown to saturation over night in 6ml LB media containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline. The cells were pelleted by centrifugation using a J-6M swinging bucket centrifuge (Beckman) for 15 minutes at 3000 rpm in 15 ml sterile Falcon tubes. Pelleted cells were resuspended in 500 µl of a buffer solution containing 15 mM Tris (pH 8.0), 10 mM EDTA (pH 8), and 5 µg RNase. Resuspended cells were lysed by adding 500 µl of the lysis buffer containing 0.2 M NaOH and 1% SDS. After 5 minutes incubation at room temperature, 500 µl of an ice cold 3 M potassium acetate solution (pH 5.5) was slowly added and the mixtures were incubated on ice for 10 minutes. The cloudy suspensions were cleared by centrifugation for 10 minutes at 14000 rpm in a benchtop centrifuge. The supernatants were transferred into 15 ml sterile

Falcon tubes each containing 1.6 ml ice cold absolute isopropanol and incubated on ice for 30 minutes. The precipitated DNA was centrifuged in 1.5 ml Eppendorf tubes at 14000 rpm for 15 minutes. The DNA pellets were washed with 1 ml of a 70% ethanol solution and re-pelleted by centrifugation in a benchtop centrifuge at 14000 rpm for 5 minutes. The pellets were briefly air dried and dissolved in 40 μ l of 10 mM Tris (pH 8) buffer. The plasmids were stored light protected at -20°C.

Transfection of Sf9 Cells with Recombinant Bacmid Plasmid

Preparation of the Sf9 Cells for Transfection

For each transfection 2 ml of cells at a density of 5×10^5 cells/ml were placed into a sterile 6-well-cell culture plate (Falcon) and incubated for one hour at room temperature.

Preparation of the Bacmid for Transfection

For each transfection 5 μ l bacmid DNA was mixed with 100 μ l of Sf-900 II SFM media in a sterile 1.5 ml Eppendorf tube (mix1). In a separate tube 100 μ l of Sf-900 II SFM media and 6 μ l of Cellfectin[®] were mixed for each transfection (mix2). Mix2 was added to mix1 and incubated light protected for 45 minutes at room temperature. The incubation allowed the Sf9 cells to attach to the surface of the cell culture plate. The over-laying media was carefully aspirated and the cells were washed with 2 ml of the Sf-900 II SFM media. After aspirating off the wash media, the Sf9 cells were immediately overlaid with the DNA solution. The 6-well-plate was transferred into a 27°C incubator for 5 hours. The transfection mixture was removed after 5 hours and the cells were overlaid with 2 ml of the Sf-900 II SFM media supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin and 292 μ g/ml L-glutamine, and 5% fetal bovine serum (FBS). The virus (P₀ virus) is harvested after 72 hours incubation at 27°C. The virus was sterile filtered using a 0.22 μ m syringe filter into a sterile 1.5 ml Eppendorf tube and stored light protected at 4°C.

Viral Amplification Protocols

Sf-900 II SFM media supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 292 µg/ml L-glutamine, and 5% FBS was used for all viral amplification and protein expression.

Procedure for Counting Sf9 Cells and Viability Determination

The viability was determined by counting the Sf9 cells under a microscope using a hemocytometer. To distinguish between dead and healthy cells, the cells were diluted 10-fold in a (1:1) mixture by volume of PBS buffer and trypan blue. Healthy cells can exclude the trypan blue dye and appear round shaped with a dark edge whereas dead cells incorporate the dye into their cell membrane and appear blue. The viability is determined by dividing the number of dead cells by the total number of healthy and dead cells.

1 L (10X) PBS, pH 7.2

10.9 g Na₂HPO₄

3.2 g NaH₂PO₄

90 g NaCl

P₁ Viral Amplification

For the P₁ amplification, 100 ml of Sf9 cells at a density of 5x10⁻⁵ cells/ml was mixed with 100 µl P₀ virus (1.8x10⁸ pfu/ml) in a 500 ml double autoclaved flask. The cells were incubated in a shaking incubator at 27°C for 7 days. The P₁ virus was harvested after the viability of the Sf9 cells dropped under 10%. The cells were centrifuged in a J-6M swinging bucket centrifuge (Beckman) for 20 minutes at 3800 rpm. The supernatant was sterile filtered using a 0.22 µm filter unit. The virus was stored light protected at 4°C.

P₂ Viral Amplification

For the P₂ amplification, 1000 ml of Sf9 cells at 5x10⁻⁵ cells/ml was mixed with 250 µl P₁ (1.8x10⁸ pfu/ml) virus in a 6 L double autoclaved flask. The cells were incubated in a shaking incubator (120 rpm) at 27°C for 7 days. The P₂ virus was harvested after the viability dropped under 10%. The cells were centrifuged in a

J-6M swinging bucket centrifuge (Beckman) for 20 minutes at 3800 rpm. The supernatant was sterile filtered using a 0.22 μm filter unit. The virus was stored light protected at 4°C.

Viral Titre Assays

Because the myosin VI protein is C-terminally tagged with YFP, the viral titre can be determined using fluorescence scanning methods. The assays were done in a 96-well plate format. 100 μl of Sf9 cells at a density of 1×10^6 cells/ml were aliquoted into each well. The first row was not infected and served as a negative control. A serial dilution of the virus from 10^{-3} to 10^{-9} was added into each row, starting from the second row by infecting all wells in one row for each dilution (Figure 21, Figure 22).

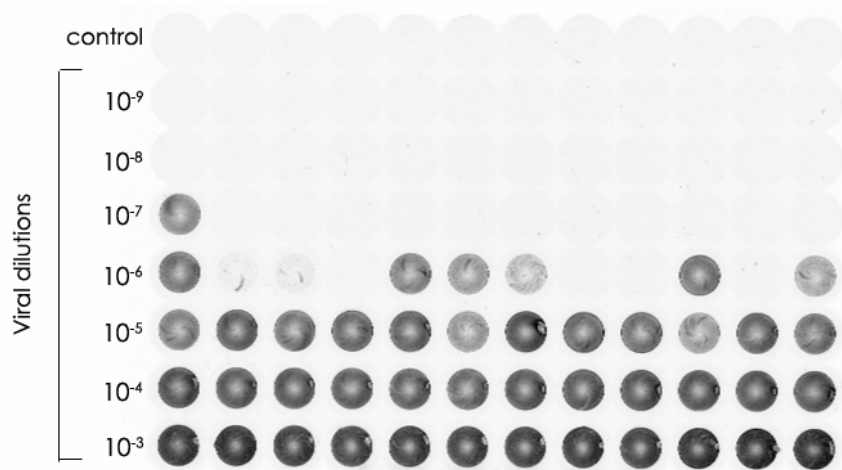


Figure 21: The fluorescence scanning of the P_0 generation viral titre assay for the CCRECC-tagged myosin VI construct in 96-well plate format. The first row is the uninfected control. The following rows are infected with increasing amounts of P_0 virus dilutions ranging from 10^{-9} to 10^{-3} .

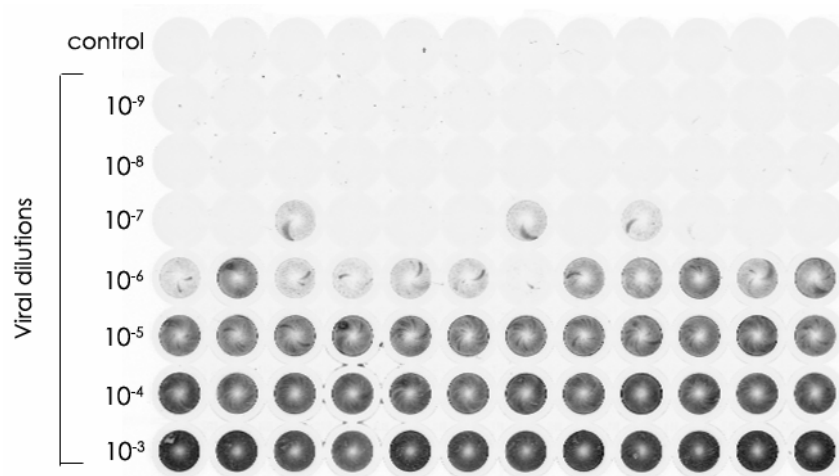


Figure 22: P₁ Generation Viral Titre Assay of the CCRECC-tagged myosin VI construct in 96-well plate format. The first row is the uninfected control. The following rows are infected with increasing amounts of P₀ virus dilutions ranging from 10⁻⁹ to 10⁻³.

The plate was placed into a nutator and incubated head-over-head shaking for 5 days at 27°C. The fluorescent protein was detected using the Typhoon 8600 Molecular Dynamics fluorescence scanner (Emission Filter: Fluorescein 526 nm, PMT 500 V, Excitation Source 532 nm green laser, focal plane +3 mm). The calculations to determine the titre were adapted from Reed, L. and H. Muench⁷¹.

FLAG-tagged Protein Purification and ReAsH Labeling of the CCRECC-tagged Myosin VI Construct

Following protocol refers to a 1 L culture of myosin VI protein expression in baculovirus infected Sf9 cells. However, the protocol can be proportionally scaled down to 20 ml culture. All steps including the centrifugation steps were carried out at 4°C. The cells were harvested by centrifugation at 3800 rpm in a Beckman swinging bucket centrifuge J-6M for 20 minutes. The supernatant was discarded and the pellet was resuspended in 50 ml lysis buffer. The suspension was carefully homogenized using a glass homogenizer. The lysed cells were pelleted in a Beckman ultra centrifuge L8-M at 45000 rpm for one hour. For batch binding, the crude lysate was incubated head-over-head shaking overnight with 3 ml Anti FLAG[®] M2 Affinity Resin. The mixture was poured into a

disposable 30 ml column. The resin was washed with wash buffer in 30 ml final volume. For the elution step the resin was resuspended in 1.5 ml of the elution buffer and incubated for one hour. The elution was completed by repeating the elution step with another 1.5 ml of the elution buffer. The protein was precipitated using 0.4 g of ammonium sulfate per milliliter of eluted sample by constant stirring for one hour. The precipitate was centrifuged in a Beckman TLX centrifuge at 37000 rpm for 10 minutes and dialyzed into 500 ml assay buffer over night in a dialysis tube with 3500 MW cut off. The buffer was exchanged next day and the protein was dialyzed for an additional 4 hours. For storage, the protein was dialyzed into 500 ml of the 50% glycerol assay buffer. Under these conditions the protein is stable for several months at -20°C.

Lysis Buffer

200 mM NaCl
4 mM MgCl₂
20 mM Imidazole, pH 7.5
0.5 mM EDTA
1 mM EGTA
0.5% Igepal
7% Sucrose
1 mM PMSF
10 µg/ml Aprotinin
10 µg/ml Leupeptin
5 mM DTT
2 mM ATP

Wash Buffer

150 mM KCl
20 mM Imidazole, pH 7.5
5 mM MgCl₂
1 mM PMSF
10 µg/ml Aprotinin
10 µg/ml Leupeptin
0.5 mM TCEP

1 mM EDTA
1 mM EGTA
3 mM ATP

Elution Buffer

150 mM KCl
20 mM Imidazole, pH 7.5
5 mM MgCl₂
1 mM PMSF
10 µg/ml Aprotinin
10 µg/ml Leupeptin
0.5 mM TCEP
1 mM EDTA
1 mM EGTA
3 mM ATP
0.2 mg/ml FLAG[®] peptide

Assay Buffer

25 mM KCl
25 mM Imidazole, pH 7.5
1 mM EGTA
4 mM MgCl₂
0.5 mM TCEP

The purified protein was labeled with the ReAsH dye using a (1:1) protein to dye ratio at room temperature for 2 hours in the labeling buffer. The excess dye was quantitatively removed by incubating the protein with 20-50 Mesh Bio-Beads[®] SM-2 over night. The mixture was centrifuged in a table top centrifuge at 14000 rpm for 1 minute. The supernatant was removed into a sterile 1.5 ml tube.

Labeling Buffer

150 mM NaCl
50 mM Hepes pH7.4
1 mM TCEP
1 mM βME

Protein Purification and Cy5 Dye Labeling of the KKCK-tagged Myosin VI Construct

FLAG-tagged Protein Purification

FLAG-tagged protein purification, fluorophore labeling and cation exchange column (MonoS) purification (FPLC) to remove unreacted dye were performed same day. The FLAG tag purification buffers were essentially the same as described for the CCRECC-tagged construct. Except 5 mM DTT was used in the lysis buffer and 0.5 mM TCEP in the wash- and elution buffer instead of 10 mM DTT. The protein was labeled immediately after the first elution from the Anti FLAG® M2 Affinity Resin column and purified over a MonoS cation exchange column (Pharmacia) to remove excess dye.

Cy5 dye Labeling and Cation Exchange Chromatography

The eluted protein from the FLAG column was diluted 3-fold in the assay buffer without DTT. Because the myosin VI construct was C-terminally tagged with the YFP protein, the protein concentration was determined using an extinction coefficient of $\epsilon_{\text{YFP}} = 94500 \text{ M}^{-1}\text{cm}^{-1}$ at 514 nm⁷⁰. A (1 : 0.5) ratio of YFP to Cy5™ maleimide monofunctional dye was used to label the KKCK-tagged myosin VI protein. The protein was labeled at room temperature and the reaction was quenched after 20 minutes in 10 mM DTT. At this stage final potassium chloride concentration in the buffer was 60 mM. To ensure binding to the MonoS column, the labeled protein was carefully diluted 20-fold into the 0 salt buffer leading to 3 mM final (monovalent) salt concentration. The protein was loaded at 0.5 ml/min onto the MonoS column that was previously equilibrated with the low salt buffer. The bound protein was then washed with 15 column volumes with the low salt buffer at 1 ml/min. To elute the protein, a 10 mM to 500 mM KCl gradient was used over 40 ml at 1 ml/min. It was critical to prepare and titrate all buffers to pH 6.1 on the same day of the MonoS column purification because the protein binding to the MonoS column and elution was highly sensitive to the accurate pH of the buffers. The protein containing fractions were pooled and dialyzed over night into the assay buffer with 50% glycerol for

storage purposes. The dialysis buffer was exchanged and the protein was dialyzed for another 4 hours. The protein was stored at -20°C.

0 Salt Buffer	Low Salt Buffer	High Salt Buffer
0 mM KCL	10 mM KCL	500 mM KCL
25 mM MES, pH 6.1	25 mM MES, pH 6.1	25 mM MES, pH 6.1
1 mM EGTA	1 mM EGTA	1 mM EGTA
1 mM EDTA	1 mM EDTA	1 mM EDTA
10 mM DTT	10 mM DTT	10 mM DTT
10 % Glycerol	10 % Glycerol	10 % Glycerol

In vitro Motility Assays

The sliding filament assay was utilized to test the functionality of the recombinant, purified proteins³⁹. In this assay the motor proteins are attached to the surface via antibody interactions. The YFP moiety was used to bind the myosin VI construct to the anti-GFP antibody coated surface. TRITC phalloidin actin (excitation: 557 nm; emission: 576 nm) was then excited and visualized while being moved by the motor proteins. The sliding filament assay was performed with purified myosin VI as well as with the crude lysate obtained from mutant protein test expressions. The assays for purified protein and crude lysate were identical except for the crude cell lysate, the flow cell was washed three times after loading the crude lysate.

Flow Cell Preparation

The cells were prepared by placing a microscope slide on a flat surface and placing two pieces of double sided tape along the length of the slide. The two pieces were about 5 mm apart. Next a nitrocellulose-coated cover slip was placed over the tape. The nitrocellulose-coated cover slips were prepared by

pre-incubating the cover slips with a 0.1% nitrocellulose solution. The nitrocellulose solution was prepared by diluting 2% collodion solution 20-fold in iso-amylacetate, and letting them air dry. The reagents were added by pipetting them into the opening at one end of the channel created by the tape and cover slip (Figure 23).

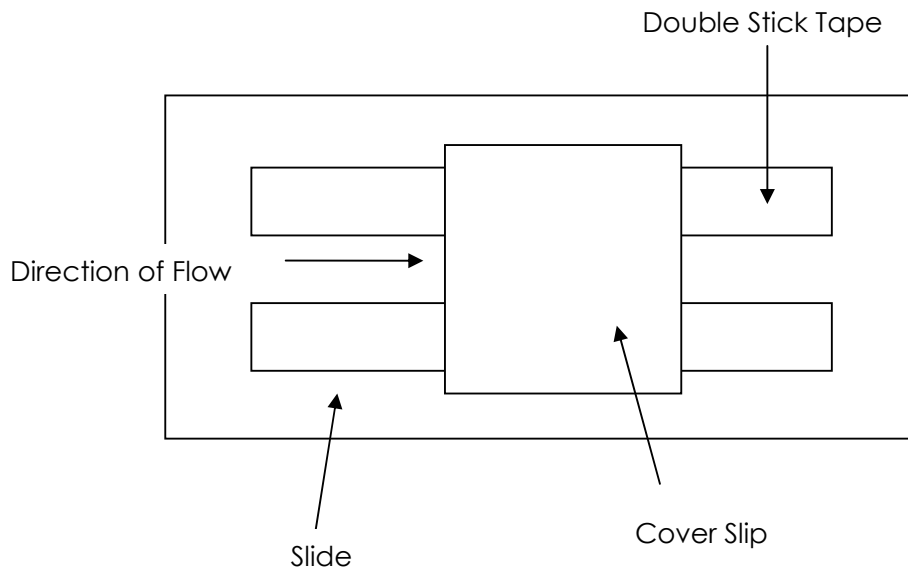


Figure 23: Illustration of the flow cell for *in vitro* motility assays.

The order of addition was as listed below. 2 minutes of incubation time was allowed after each addition.

1. 7 μ l Anti-GFP anti body
2. 15 μ l Assay buffer-BSA
3. 15 μ l Purified protein dilution or crude lysate
4. 15 μ l Assay buffer-BSA
5. 15 μ l (0.1 μ M) TRITC phalloidin actin
6. 15 μ l Motility buffer

The data was collected immediately after adding the motility buffer. Purified myosin diluted into assay buffer a concentration range of 1 nM to 100 nM. The crude lysate for testing the initial functionality of the mutants was applied undiluted. Actin was prepared by diluting a 10 μ M stock 100-fold in the assay buffer. The anti-GFP antibody was diluted 10-fold from a 0.5 μ g/ μ l stock in the

assay buffer. The time lapse movies were made at one frame per second with 60 frames in total and analyzed using the program NIH-Image.

Assay Buffer

25 mM KCl

25 mM Imidazole, pH 7.5

1 mM EGTA

4 mM MgCl₂

10 mM DTT

Assay Buffer-BSA

25 mM KCl

25 mM Imidazole, pH 7.5

1 mM EGTA

4 mM MgCl₂

10 mM DTT

1 mg/ml BSA

Motility Buffer

90 µl Assay buffer

2 µl (0.1 M) ATP

2 µl (4.5 µM) Wild type CaM

2 µl Glucose [0.45% (w/v)] / β-Mercaptoethanol [0.25% (v/v)]

2 µl (22 µg/ml) Glucose oxidase

2 µl (36 µg/ml) Catalase

1 µl (1 mM) Creatine phosphate

1 µl (0.1 mg/ml) Creatine phosphokinase

Single Molecule In vitro Motility Assays

Flow Cell Preparation

The flow cell geometry and the assay buffers were essentially the same as in *in vitro* motility assays except NLAf 21 cover slips (VA Optical Labs) were used for

single molecule assays instead of nitrocellulose treated glass cover slips. Different approaches were pursued to suspend the actin filaments in the flow cell. The most efficient way was found to be the suspension of the actin filaments using 90 nm streptavidin-coated beads.

Suspension of Actin Filaments via 90 nm Spherical Beads

A (1:150) dilution of streptavidin-coated, spherical polystyrene beads of 90 nm diameter in the AB buffer was used to prepare a flow cell with suspended actin filaments. The order of addition was as follows:

1. 15 μ l (1 mg/ml) Biotin-BSA
2. 15 μ l (1:150) Streptavidin-coated beads in assay buffer
3. 15 μ l Assay buffer-BSA
4. 15 μ l (0.1 μ M) TRITC phalloidin actin
5. 15 μ l Motility buffer

2 minutes incubation time was allowed between each step.

Motility Buffer

89 μ l Assay buffer
4 μ l (1 mM) ATP
2 μ l (4.5 μ M) wild type CaM
11 μ g/ml Glucose oxidase
18 μ g/ml Catalase
1 mM Creatine phosphate
0.26% (w/v) Glucose
0.13% (v/v) β -mercaptoethanol
0.1 mg/ml Creatine phosphokinase
1% Triton-X 100
1 μ l (77 μ M) Phalloidin

Actin Polymerization in the Flow Cell

Biotinylated actin filaments were thoroughly sheared by vigorous pipetting and the obtained actin seeds were used to sparsely coat the flow cell surface. 20 μL of unbiotinylated G-actin (10 μM) was supplemented with 1 mM ATP to induce polymerization and was immediately flown into the flow cell. The G-actin monomers polymerized then from the surface attached actin seeds. The order of addition was as follows:

1. 15 μl (1 mg/ml) Biotin-BSA
2. 15 μl Assay buffer
3. 15 μl (0.5 mg/ml) NeutrAvidin
4. 15 μl Assay buffer-BSA
5. 15 μl Sheared biotinylated actin (10 nM)
6. 15 μl Assay buffer
7. 15 μl G-actin (10 μM) (10 minutes incubation)
8. 15 μl Assay buffer
9. 15 μl (0.77 μM) TRITC phalloidin
10. 15 μl Assay buffer-BSA
11. 15 μl Motility buffer

Two minutes incubation time was allowed between each step except for the G-actin polymerization.

Suspension of Actin Filaments via NEM-treated myosin II

N-Ethylmaleimide (NEM) treated myosin II is trapped in the rigor state with actin. This fact was exploited to firmly attach the actin filaments to the myosin II catalytic heads and thus stretch the filaments between myosin heads on the surface. Myosin S1 is roughly 65 \AA wide and 40 \AA thick. Thus, the actin filaments bound to the NEM treated myosin II filaments in the rigor state will be elevated from the surface.

NEM-myosin II preparation

600 μM (23.2 mg/ml) rabbit skeletal full-length myosin II (a kind gift from Roger Cooke, UCSF) was diluted in 5.2 ml BE buffer and incubated 10 minutes on ice.

The protein was centrifuged 10 minutes in a Beckman J-6M swinging bucket centrifuge at 9000 rpm and the supernatant was discarded. The pellet was dissolved in (2X) 400 μ l LB buffer and 400 μ l BE buffer and incubated 10 minutes on ice. 12 μ l (0.1 M) freshly prepared NEM in the BE buffer was added to the dissolved pellet and incubated 20 minutes at room temperature. 9.6 ml of the SB buffer was added and the mixture was incubated one hour on ice. The NEM treated myosin II protein was pelleted by centrifugation at 9000 rpm for 10 minutes in a Beckman J-6M swinging bucket centrifuge. The pellet was removed into a 1.5 ml Eppendorf tube and centrifuged 10 minutes at 14000 rpm in a benchtop centrifuge at 4°C. The supernatant was pipetted out and the pellet was soaked with 500 μ l HSAB buffer to dissolve the myosin thick filaments. The protein yield was 10 - 30 mg/ml.

BE Buffer

0.1 mM NaHCO₃

0.1 mM EGTA

2X LB Buffer

20 mM Imidazole pH 7.4

1 M KCl

4 mM MgCl₂

SB Buffer

0.1 mM NaHCO₃

0.1 mM EGTA

2 mM MgCl₂

7 mM DTT

HSAB Buffer

1 X AB-buffer

1 mM DTT

175 mM KCl

The order of addition into the flow cell preparation was as follows:

1. 15 μ l NEM myosin II full-length protein
2. 15 μ l Assay buffer-BSA
3. 15 μ l (0.1 μ M) TRITC phalloidin actin
4. 15 μ l Assay buffer-BSA
5. 15 μ l Motility buffer

Two minutes incubation time was allowed between each step.

Microscope Set Up

Excitation source beams at 532 nm (Coherent, Inc.) and 633 nm (JDS Uniphase) were combined by a dichroic mirror, and expanded to 7 mm diameter. These sources were focused (focus length = 500 mm) on the back focal plane of an Olympus 1.65 NA 100x TIRF objective, via a laser line dichroic on a linear translation stage that allows microscope operation in either epi-fluorescence or TIRF modes. The reflected light exiting the back aperture of the objective was directed on a quadrant photodiode, to provide a signal for a focus feedback loop that clamped the distance between the objective and sample using an electrostrictive actuator (Newport, Inc.). Fluorescence emission was collected by the objective and passed through a dual-view apparatus that allowed simultaneous imaging of the Cy5 and Cy3 channels on a single EMCCD camera (Figure 24).

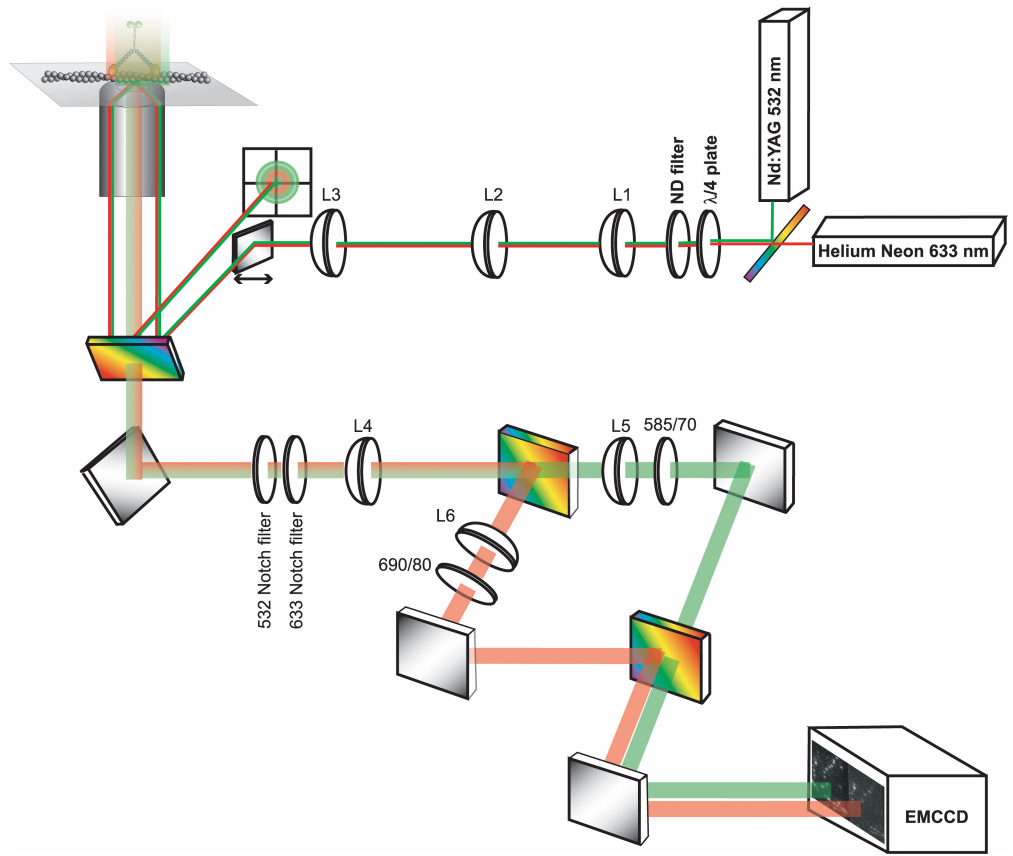


Figure 24: Schematic drawing of the microscope for dual color imaging of single fluorophores.