5 APPENDIX: Protocols

5.1 F-Actin polymerization and labeling

Materials:

- G-actin (Cytoskeleton, AKL99 1mg), reconstituted to 10 mg/ml (233 µM) with 100 µl of milliQ distilled water
- Actin polymerization buffer (Cytoskeleton, BSA02)
- DTT 1M
- Rhodamine Phalloidin 250 µM
- MilliQ distilled water

Methods:

G-actin polymerization in F-actin

In a 0,5 ml tube mix 69 µl of milliQ distilled water, 10 µl actin polymerization buffer, 20 µl G-actin stock and 1 µl DTT 1M. Put on ice for > 1 hour.

F-actin rhodamine phalloidin labeling

In a new 0,5 ml tube take 25 µl of polymerized F-actin and add 19,5 µl of milliQ distilled water, 2, 5 µl of actin polymerization buffer, 1 µl of DTT 1M and 2 µl of rhodamine phalloidin. Leave on ice overnight.

5.2 Bio-HMMVb/QD 655 nm conjugation

Materials:

- Bio-HMMVb 0,2 µM
- Streptavidin-QD 655 nm (Life Technologies Q10123MP) 0,2 µM
- Mary's Buffer 1x : 0,5 M NaCl
  10 mM MOPS
  0,1 mM EGTA
  3 mM NaN_3

- D-Biotin 10 mg/ml in Mary's Buffer 1x
- MCDα Buffer: 195,4 µl Mary's buffer 1x
  2 µl α-casein 10 mg/ml
  2 µl calmodulin 200 µM
  0,6 µl DTT 1M

Methods:

In a <0,5 ml tube mix 1 µl Bio-HMMVb 0,2 µM, 1 µl of streptavidin-QDs 0,2 µM and 2,5 µl of MCDα Buffer. Put on ice for > 30 minutes. Just before imaging put 0,5 µl of D-Biotin 10 mg/ml and wait at least 10 minutes before flux into the imaging chamber.

5.3 In vitro motility assay

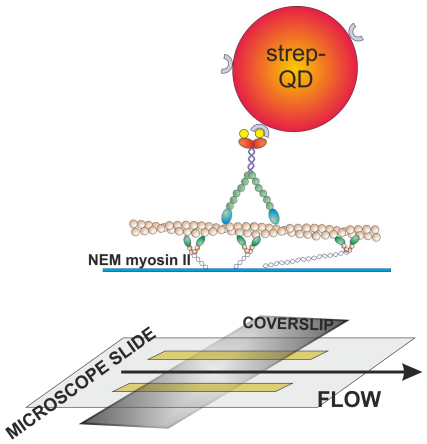


Figure 65 Pictrure of a ~ 20 µl flow chamber: take a glass coverslip 170 µm thick, clean it carefully with pure ethanol and dry it with compressed nitrogen, paying attention in not to leave any residues on the glass surface. Smear one surface of the coverslip with 1 % nicrocellulose (in pentilacetate) and wait for it to be completely dry. Take the microscope slide, clean it carefully with pure ethanol, and put two stripes of double sticky tape as shown in picture. By handlying the coverslip with tweezers close the chamber, as shown in the picture, with the nitrocellulose layer facing the inside of the chamber.

Materials:

- Flow chamber (Fig. 65)

- 4x Motilitiy Buffer (ph 7,4), filter sterilized:
  80 nM MOPS
  4 mM $MgCl_2$
  0,4 mM EGTA
- 1x Motility Buffer +50 mM KCl (MB+KCl), filter sterilized:
  28 ml milliQ H_2O
  10 ml 4 x Motility Buffer
  2 ml KCl 1 M
- MBα Buffer :
  198 µl MB+KCl
  2 µl α-casein 10 mg/ml
- Imaging Buffer (IB),make it fresh every ~4 hours since enzymes deteriorate:
  908 ml MB+KCl
  40 ml glucoseoxidase 5 mg/ml
  10 µl catalase 5 mg/ml
  10 µl α-casein 10 mg/ml
  12 µl glucose 250 mg/ml
  20 µl DTT 1 M
- IB-calm Buffer:
  198 µl IB
  2 µl calmodulin 200 µM
- ATP-mix:
  2 µl CPK 10 mg/ml (prepared fresh every day)
  1 µl CP 1M (prepared fresh avery day)
  2 µl calmodulin 200 µM
  Tot µl ATP at the wanted conc.
  195-tot ATP µl IB
- HIS 2x (ph 7,5):
  1 M KCl
  40mM KPi
  2 mM EGTA
  4 mM $MgCl_2$
- NEM-Myosin II(20 mg/ml) [116]
- Bio-HMMVb/QD 655 nm conjugated (see prot.5.2)
- Rhodamine-phalloidin labeled F-actin stock (see prot.5.1)

Methods:

1) Prepare all buffers and leave everything on ice

2) Build flow chamber with 1% nitrocellulose and leave on ice (all following operation have to be carried out on ice)

3) Flux 1 volume of NEM-Mysoin II 2mg/ml (in HIS 2x), wait 1' (sharp)

4) Wash with 3 volumes of MBα

5) Dilute labeled F-actin stock 1000 times just before use (1 µl labeled F-actin stock, 10 µl DTT 1M, 989 µl MB+KCl) and mix gently without pipetting

6) Flux diluted actin (mix gently before fluxing since actin precipitate very rapidly), wait 2'

7) Wash with at least 4 volumes of IB and watch if the filaments attached to the coverslip surface (Exc. 532 nm/Em. 580)

8) Dilute the 5µl Bio-HMMVb/QD 655 nm conjugated stock prepared before following prot.5.2 in 95 µl IB

9) Prepare 19 µl of IB-calm + 1 µl Bio-HMMVb/QD 655 diluted in IB and flux it into the chamber

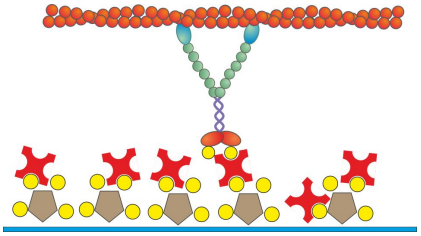
10) Watch the chamber. (For QDs: Exc. 488 nm, Em. 655 nm)

Since ATP is not present yet myosin should attach to actin filaments and stay still, therefore you should QDs stuck on actin filaments.

11) Prepare 19 µl of ATP-mix (at the desired ATP concentration) + 1 µl Bio-HMMVb/QD 655 diluted in IB and flux it into the chamber

12) Close the chamber with silicon and run for watching myosins moving along actin filaments!

5.4 In vitro Glyding assay



Materials:

For not detailed buffers see protocol 5.3

- Flow chamber (Fig.65)
- Biotinilated-BSA 1mg/ml (diluted in MB-KCl)
- Streptavidin 1mg/ml
- MB+KCl
- MBα
- Imaging Buffer (IB)
- ATP-mix-glyding:
  200 µl IB
  2 µl ATP 100 mM
  2 µl calmodulin 200 µM
- Purified Myosin stock
- Rhodamine-phalloidin labeled F-actin stock (see prot.5.1)

Methods:

1) Build chamber wih 1% nitrocellulose (Fig.65)

2) Flux biotinilated BSA 1 mg/ml, wait 3'

3) Wash with 3 volumes of MB+KCl

4) Flux streptavidin 1 mg/ml, wait 5'

5) Wash with 4 volumes of MB+KCl

6) Flux one volume of purified myosin at the desired concentration, wait 5' (sharp)

7) Wash with 4 volumes of biotinilated BSA 1 mg/ml

8) Dilute labeled F-actin stock 1000 times just before use (1 µl labeled F-actin stock, 10 µl DTT 1M, 989 µl MB+KCl) and mix gently without pipetting

9) Flux diluted actin (mix gently before fluxing since actin precipitate very rapidly), wait 2'

10) Watch very rapidly

11) Wash with 4 volumes of IB

12) Add ATP-mix-glyding

13) Close the chamber with silicon and watch

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