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Fluorescent Labeling of Myosin V for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays

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Abstract

Polarized total internal reflection fluorescence microscopy (polTIRFM) can be used to detect the spatial orientation and rotational dynamics of single molecules. polTIRFM determines the three-dimensional angular orientation and the extent of wobble of a fluorescent probe bound to the macromolecule of interest. This protocol describes how to exchange bifunctional rhodamine–calmodulin (BR-CaM) for wild-type calmodulin (WT-CaM) on the lever arm of myosin V. BR-CaM is exchanged at low stoichiometry (~0.4 BR-CaM per double-headed myosin V) to obtain myosin V molecules with one BR-CaM and to limit the proportion of myosin V molecules with two or more probes. The stoichiometry is very sensitive to the concentration of calcium during the exchange reaction. The labeled myosin V can subsequently be used for investigating the motility of myosin V in vitro with a polTIRFM processive motility assay, which is performed on substrate-attached actin.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPE: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

Bovine serum albumin (BSA) standards and other reagents for Bradford assay (or other protein colorimetric assay)

$CaCl_2$ (31.34 mM)

This concentration can be obtained from five serial 2× dilutions of 1.003 M traceable standard $CaCl_2$ (Sigma-Aldrich/Fluka 21114) into H_2O.

Calmodulin (CaM) molecules

- BR-CaM (10 μM), prepared as in **Fluorescent Labeling of Calmodulin with Bifunctional Rhodamine** (Beausang et al. 2012a)
- Wild-type (WT)-CaM from chicken (3.9 mg/mL), expressed in bacteria (Putkey et al. 1985)

EGTA (200.0 mM at pH 7.5) (>99% pure; Sigma-Aldrich 03778)

Exchange buffer for myosin labeling <R>

Myosin V from chick brain, purified as described by Cheney (1998)

Storage buffer (100 mg/mL sucrose, prepared in exchange buffer for myosin labeling)

Equipment

Centrifugal filter device (100-kD cutoff; Pall Corporation Nanosep OD100C33)

Centrifuge

Dialysis unit (10-kD cutoff; Thermo Scientific/Pierce Slide-A-Lyzer MINI 69570)

Heat block preset to 25°C

METHOD

1. Chill exchange buffer to 4°C for use in dialysis.
2. Dialyze 100 μL of WT-CaM and 100 μL of BR-CaM (in separate dialysis tubes) against 900 mL of exchange buffer at 4°C.
3. After 1 h, replace the exchange buffer. Dialyze overnight at 4°C.
4. On the following day, determine the concentrations of WT-CaM and BR-CaM (e.g., using the Bradford assay).
5. Combine 100 nM of myosin V, 100 nM of BR-CaM, and 900 nM of WT-CaM in a total volume of 200 μL of exchange buffer.
6. Incubate the sample for 5 min at 25°C.
7. Add 6.6 μL of 31.34 mM $CaCl_2$ to the sample.

   The concentration of $CaCl_2$ in the sample is now 1.00 mM.
8. Incubate the sample for 10 min at 25°C.
9. Add 4.2 μL of 200.0 mM EGTA to the sample.

   The EGTA, now at a concentration of 4 mM, will stop the exchange reaction.
10. Incubate the sample for 10 min at 25°C, and then place the reaction tube on ice.
11. Remove the excess CaM by filter-centrifugation as follows:
    - **i.** Transfer the ~200-μL reaction mixture to the centrifugal filter device, and centrifuge at 5000*g* for 3–5 min at 4°C.
    - **ii.** Measure the volume of the filtrate. Add this volume of storage buffer to the retained solution to restore it to the original volume.
    - **iii.** Agitate the solution carefully with a pipette in order to resuspend any myosin that is caught in the filter membrane. Take care not to damage the filter membrane.
    - **iv.** Repeat the concentration and redilution procedure (Steps 11.i–11.iii) four times to wash any unbound CaM through the filter membrane.
12. Flash-freeze small aliquots of purified myosin at −80°C. Store the aliquots for up to 6 mo.

RELATED INFORMATION

A method is also available for **Preparation of Filamentous Actin for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays** (Beausang et al. 2012b). Further information on analysis of single molecules using polTIRFM can be

found in **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy (pol-TIRFM)** (Beausang et al. 2012c).

RECIPE

Exchange Buffer for Myosin Labeling

20 mM imidazole (pH 7.6) 25 mM KCl

2 mM $MgCl_2$

1.0 mM EGTA (accurate concentration is important)

5 mM dithiothreitol (DTT)

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