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The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Processive Motility Assay for Myosin V

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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 the processive motility assay for investigating the motility of myosin V in vitro. Biotin-Alexa actin filaments are fixed to a slide by biotin/streptavidin linkages and aligned with the microscope x-axis by fluid flow. The orientation of a rhodamine–calmodulin (CaM) probe bound to a single myosin V molecule is determined as it moves along an actin filament. Excess wild-type calmodulin (WT-CaM) is present in the buffer solution to replenish lost CaM from the myosin lever arm. The techniques for myosin V should be generally applicable to other single-molecule experiments where angular changes have an important mechanistic role in their biological function.

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.

RECIPES: 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

Biotin-Alexa actin (1 μM), prepared as in Preparation of Filamentous Actin for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays (Beausang et al. 2012a)

Prepare a 1:10 dilution of biotin-Alexa actin in M5+ daily.

Biotinylated bovine serum albumin (BSA; 1 mg/mL) (Sigma-Aldrich A8549), prepared monthly in M5 buffer

M5 buffer <R>

M5+ <R>

Motility solution <R>

Streptavidin (5 mg/mL; Sigma-Aldrich S4762), prepared monthly in M5 buffer

Prepare a 1:10 dilution of streptavidin in M5+ daily.

Equipment

Filter paper (2-ply; Kettenbach 30761 or similar)

Flow chamber, prepared as in Construction of Flow Chambers for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays (Beausang et al. 2012b)

Pipette tip (cut; see Step 5)

polTIRFM and analysis software

For details, see the section entitled The Principles of polTIRFM in Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) (Beausang et al. 2012c).

METHOD

When possible, all solutions should be prepared using 0.2-μm-filtered, deionized water in a clean hood while wearing gloves to prevent sample contamination.

1. Use a flow chamber to flow 20 μL of 1 mg/mL biotinylated BSA. Incubate for 5 min.
2. Rinse the chamber with 50 μL of M5+.
3. Flow 20 μL of 0.5 mg/mL streptavidin. Incubate for 2 min.
4. Rinse the chamber with 100 μL of M5+.
5. Use a cut pipette tip to flow 20 μL of the 1:10 dilution of biotin-Alexa actin. Wait 30 sec.

   The cut pipette tip minimizes the shearing of F-actin. To obtain well-aligned actin filaments, use 2-ply filter paper to increase the flow rate during the addition and washout of the actin.
6. Rinse with 50 μL of M5+.
7. Flow 20 μL of motility solution. Record and analyze the data using the polTIRFM and analysis software as specified in The Acquisition and Analysis of Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Data (Beausang et al. 2012d).

RELATED INFORMATION

An assay that examines labeled actin filament translocation on a myosin-coated slide is described in The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Twirling Filament Assay (Beausang et al. 2012e).

RECIPES

M5 Buffer

20 mM HEPES (pH 7.6)

2 mM $MgCl_2$

25 mM KCl

1 mM EGTA

M5+

10 mM DTT (prepare 1 M DTT stock in M5 buffer daily)

100 μg/mL wild-type calmodulin (WT-CaM)

Prepare daily in M5 buffer.

Motility Solution

10–1000 pM bifunctional rhodamine-calmodulin (BR-CaM)-labeled myosin V, prepared as in Fluorescent Labeling of Myosin V for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays (Beausang et al. 2012)

2.5 mg/mL bovine serum albumin (BSA; Sigma-Aldrich A0281)

100 mM DTT (prepare 1 M DTT stock in M5 buffer daily)

100 μg/mL wild-type calmodulin (WT-CaM)

1–200 μM ATP

Prepare daily in M5 buffer.

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