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The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Twirling Filament Assay

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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 twirling filament assay, so named because actin sometimes twirls about its own axis as it is translocated by myosin. A gliding filament assay is constructed in which a sparsely labeled actin filament (0.3% of the actin monomers contain 6'-iodoacetamidotetramethylrhodamine [IATR]) is translocated by a field of unlabeled myosin V fixed to the surface. The polTIRFM twirling assay differs from a standard gliding filament assay in that full filaments are not visible, but rather individual fluorophores are spaced along each filament. The goal is to investigate possible rotational motions of the actin filament about its axis (i.e., twirling) by measuring the spatial angle of the fluorescent probe as a function of time. Successful assays contain microscopic fields of approximately 50 isolated points of fluorescence that move across the field in the presence of ATP. Actin is usually translocated by more than one myosin molecule, depending on the filament length and the myosin surface density. Sparsely labeled filaments are required because the orientation of only one probe can be resolved at a time.

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

ATP (2 mM)

Dilute ATP daily in M5 buffer from 100 mM stock (stored at -20°C , thawed shortly before use).

Bovine serum albumin (BSA; 5 mg/mL) (Sigma-Aldrich B2518), prepared monthly in M5 buffer

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

F-actins

- Labeled (0.3%) with 6'-IATR (1 μM) (see Preparation of Filamentous Actin for Polarized Total Internal Reflection Fluorescence Microscopy [polTIRFM] Motility Assays [Beau-sang et al. 2012a])

Prepare a 1:500 dilution of 0.3% 6'-IATR-labeled F-actin in M5+ daily.

- Unlabeled (70 μM stock) (prepared similarly)

Prepare a 1:20 dilution of unlabeled F-actin in M5+ daily. Shear the actin by rapidly passing it through a 26-gauge needle 10 times.

M5 buffer <R>

M5+ <R>

Motility buffer for actin <R>

Myosin V, unlabeled (~0.2 mg/mL)

Poly-L-lysine HBr (1.0 mg/mL; MP Biomedicals 71120)

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), but without poly (methyl methacrylate) (PMMA)

Pipette tip (cut; see Step 9)

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- μ m-filtered, deionized water in a clean hood while wearing gloves to prevent sample contamination.

1. Using a flow chamber prepared without PMMA, flow 20 μ L of poly-L-lysine. Incubate for 1 min.
2. Flow 20 μ L of M5+.
3. Flow 20 μ L of unlabeled myosin V. Incubate for 2 min.
4. Flow 20 μ L of the BSA dilution twice. Incubate for 1 min.

This blocks exposed glass or poly-L-lysine.

5. Flow 20 μ L of diluted, sheared, unlabeled F-actin. Incubate for 1 min.

This blocks the inactive myosin heads.

6. Repeat Step 5.
7. Flow 20 μ L of 2 mM ATP.

This releases the unlabeled actin from the active myosin heads.

8. Immediately flow 20 μ L of M5+ twice.

This washes out the ATP and the unbound actin.

9. Use a cut pipette tip to flow 20 μ L of the 0.3% 6'-IATR-labeled F-actin dilution. Incubate for 1 min.

The cut pipette tip minimizes the shearing of the actin.

10. Flow 20 μL of motility buffer for actin to initiate gliding. Record the data using the polTIRFM and analysis software as described in The Acquisition and Analysis of Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Data (Beausang et al. 2012d).

An assay that examines myosin movement along a stationary actin filament is described in The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Processive Motility Assay for Myosin V (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 $\mu\text{g/mL}$ wild-type calmodulin (WT-CaM)

Prepare daily in M5 buffer.

Motility Buffer for Actin

5–50 μM ATP

10 mM phosphocreatine (stocks stored at -20°C and thawed daily; Sigma-Aldrich P7936)

0.3 mg/mL creatine phosphokinase (prepared daily from powder; Sigma-Aldrich C3755)

50 mM DTT (prepare 1 M DTT stock in M5+ daily)

Prepare daily in M5+.

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