Published in final edited form as:
Cold Spring Harb Protoc. ; 2012(5): . doi:10.1101/pdb.prot069377.

Preparation of Filamentous Actin for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays

John F. Beausang, **Yujie Sun**, **Margot E. Quinlan**, **Joseph N. Forkey**, and **Yale E. Goldman**

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. In this protocol, filamentous actin (F-actin) is polymerized from purified, monomeric actin (G-actin) for use in polTIRFM motility assays in which actin interacts with myosin. The procedures include (1) the preparation of unlabeled F-actin from G-actin; (2) the preparation of F-actin that is sparsely labeled with 6′-IATR (6′-iodoacetamidotetramethylrhodamine); and (3) the preparation of F-actin with a combination of unlabeled, biotinylated, and rhodaminelabeled monomers. Rhodamine–phalloidin actin, also used in polTIRFM assays, can be prepared using a procedure similar to the one for unlabeled 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.

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

Actins—

- Alexa-actin, prepared by labeling G-actin with Alexa Fluor 647 as described for AEDANS-actin inForkey et al. (2003)
- Biotin-labeled actin (Cytoskeleton, Inc. AB07)
- G-actin, isolated and purified from rabbit muscle (Pardee and Spudich 1982)
- Rhodamine-actin, prepared by labeling G-actin with 6′-IATR (Corrie and Craik 1994)

  All G-actin and labeled actin monomers should be stored in G-actin buffer.

F-actin buffer (4×) <R>

G-actin buffer <R>

Phalloidin (Invitrogen P3457)

METHOD

1. Combine labeled and unlabeled G-actin (or unlabeled G-actin only) at the desired ratio (e.g., see Box 1 and Box 2). Mix well.

2. To the monomer solution from Step 1, add H_2O, 4× F-actin buffer (to a final concentration of 1×), and 1.1 μM phalloidin (in that order) so that the final concentration of actin monomers is 1 μM.
3. Gently mix the solution for ~10 sec.
4. Incubate the mixture for 10 min at room temperature.
5. Transfer the mixture onto ice, and store it for up to 1 mo at 4°C.

RELATED INFORMATION

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 (polTIRFM)** (Beausang et al. 2012a).

Protocols are also available for **Fluorescent Labeling of Calmodulin with Bifunctional Rhodamine** (Beausang et al. 2012b) and **Fluorescent Labeling of Myosin V for Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Motility Assays** (Beausang et al. 2012c). The resulting labeled compounds can be used in polTIRFM motility assays.

RECIPES

F-Actin Buffer (4×)

300 mM KCl

10 mM $MgCl_2$

40 mM HEPES (pH 7.0–7.2 at 20°C)

G-Actin Buffer

2 mM Tris-Cl (pH 8.0 at 20°C)

0.2 mM $CaCl_2$

0.2 mM ATP (added fresh)

0.5 mM dithiothreitol (DTT; prepared from powder daily)

Acknowledgments

This work was funded by National Institutes of Health grants AR26846 and AR51174 to the Pennsylvania Muscle Institute and by the National Science Foundation (NSF) NSEC Nano/Bio Interface Center (NBIC) DMR 04-25780. J.F.B. is supported by an NSF IGERT fellowship through the NBIC DGE 0221664. We thank Drs Philip C. Nelson and Jody A. Dantzig-Brody for review and useful comments on the manuscript.

REFERENCES

1. Beausang JF, Sun Y, Quinlan ME, Forkey JN, Goldman YE. Orientation and rotational motions of single molecules by polarized total internal reflection fluorescence microscopy (polTIRFM). Cold Spring Harb Protoc. 2012a
2. Beausang JF, Sun Y, Quinlan ME, Forkey JN, Goldman YE. Fluorescent labeling of calmodulin with bifunctional rhodamine. Cold Spring Harb Protoc. 2012b
3. Beausang JF, Sun Y, Quinlan ME, Forkey JN, Goldman YE. Fluorescent labeling of myosin V for polarized total internal reflection fluorescence microscopy (polTIRFM) motility assays. Cold Spring Harb Protoc. 2012c

4. Corrie JET, Craik JS. Synthesis and characterisation of pure isomers of iodoacetamidotetramethylrhodamine. J Chem Soc Perkin Trans. 1994; 1:2967–2973.
5. Forkey JN, Quinlan ME, Shaw MA, Corrie JET, Goldman YE. Three-dimensional structural dynamics of myosin V by single-molecule fluorescence polarization. Nature. 2003; 422:399–404. [PubMed: 12660775]
6. Pardee JD, Spudich JA. Purification of muscle actin. Methods Cell Biol. 1982; 24:271–289. [PubMed: 7098993]

BOX 1. PREPARATION OF SPARSELY LABELED RHODAMINE ACTIN FILAMENTS

1. Prepare stock A (75 μM total actin) by mixing together the following:
   - 9.5 μL of G-actin buffer
   - 9.5 μL of 6.5 mg/mL G-actin
   - 1.0 μL of 33.5 μM G-actin (with 13.5% of monomers labeled with rhodamine)
2. To 10 μL of stock A, add the following ingredients in the order listed:
   - 552 μL of H_2O
   - 187.4 μL of F-actin buffer (4×)
   - 0.6 μL of 1.3 mM unlabeled phalloidin
3. Proceed with Steps 3–5 in the protocol.

The resulting 750-μL solution contains 75 mM KCl (from the F-actin buffer), 1.1 μM unlabeled phalloidin, and 1 μM actin monomers, 0.3% of which are labeled with rhodamine.

BOX 2. PREPARATION OF BIOTIN-ALEXA ACTIN FILAMENTS

1. Combine 34.7 μL of 2.4 μM biotin-actin and 15.7 μL of 26.5 μM Alexa-actin. Mix well.
2. Add the following ingredients in the order listed:
   - 324.1 μL of H_2O
   - 125 μL of F-actin buffer (4×)
   - 0.5 μL of 1.3 mM unlabeled phalloidin
3. Proceed with Steps 3–5 in the protocol.

The resulting 500-μL solution contains 75 mM KCl (from the F-actin buffer), as well as 1 μM actin in a 1:5 ratio of biotin-actin:Alexa-actin (0.17 μM biotin-actin, 0.83 μM Alexa-actin).