Published in final edited form as:

Cold Spring Harb Protoc. ; 2012(6): . doi:10.1101/pdb.prot069419.

The Acquisition and Analysis of Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Data

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. This protocol describes how to acquire polTIRFM data and then calibrate the setup. Calibration corrects for any systematic variations in beam intensity and unequal detector sensitivities and is performed for each slide after experimental data are recorded. To convert the intensities into angles, one set of $(\theta, \phi, \delta_s, \delta_f, \kappa)$ is then determined from one complete cycle of the incident intensities. This process is repeated for every cycle in the trace to measure the time dependence of rotational motions. The collection and analysis of data is similar for the processive motility assay for myosin V and for the twirling filament assay, in which a sparsely labeled actin filament is translocated by a field of unlabeled myosin V.

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.

Reagents

RhoB calibration solution (50 nM rhodamine B [laser grade; Acros 41900] and 5% dimethylformamide [DMF])

The DMF minimizes the adherence of the rhodamine to the surface.

Equipment

Flow chambers from **The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Processive Motility Assay for Myosin V** (Beausang et al. 2012a) or **The Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Twirling Filament Assay** (Beausang et al. 2012b)

polTIRFM apparatus (see Fig. 1) and analysis software (for details, see the section entitled The Principles of polTIRFM in the article **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy [polTIRFM]** [Beausang et al. 2012c])

METHOD

Data Collection

- 1 View the field of candidate molecules via an intensified charge-coupled device (ICCD) camera. Use brief, intermittent illumination to avoid bleaching. Superimpose successive images on the computer monitor with two different colors to accentuate the differences between the frames.

- 2 Select a molecule that is moving at an appropriate rate for the myosin isoform and ATP concentration by clicking over its image. Save images of the field with the initial coordinates of the selected molecule on a disk for subsequent analysis.
- 3 Via a software interface, move the piezoelectric microscope stage and steering mirror (RM in Fig. 1) to align the selected molecule with the detector(s).
- 4 Illuminate the sample successively with alternating excitation polarizations and collect the fluorescent emission. To display the intensities graphically, use weighting factors to sum the separate polarized fluorescence intensity traces into one total intensity that is relatively insensitive to probe orientation (see Forkey et al. 2005).

The intensity of the laser should be adjusted so that a typical recording period (e.g., 10 sec) consists of fluorescence signals for about two-thirds of the recording time followed by a bleach and background intensities for the remaining one-third of the measurement.
- 5 For additional molecules, repeat Steps 1–4.

Calibration

- 6 In the same flow chamber that was used to collect experimental data, record three sets of background intensity measurements, carefully avoiding fluorescent molecules (i.e., there should be no bleach).
- 7 Flow 20 μ L of RhoB calibration solution.
- 8 Take three measurements of the RhoB fluorescence intensities.
- 9 Flow 20 μ L of H₂O twice.
- 10 Measure the background again to ensure that the rhodamine molecules did not stick to the surface (i.e., the intensities should equal those from Step 6).

High background intensities remaining after washout indicate that the rhodamine is sticking to the surface, and the assumption of an isotropic distribution of the calibrating fluorophores may not be valid. If this is the case, try increasing the DMF concentration or change surfaces, e.g., to nitrocellulose or poly(methyl methacrylate) (PMMA).

- 11 Obtain calibration signals by subtracting the average background measured in Step 6 from the signal measured in Step 8.
- 12 Modify the probe model to accommodate the isotropic distribution of fluorophores in solution (see the section entitled The Principles of polTIRFM in the article **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy [polTIRFM]** [Beausang et al. 2012c]).

The main difference is that the slow wobble cone, which in this case represents the isotropic distribution of the rhodamine molecules, extends over all angles, thus eliminating parameters δ_s , θ , and ϕ . The modified probe model predicts that different polarizations have somewhat different intensities. Deviations from the expectations are caused by unequal detector sensitivity and beam intensities. The calibration factors that account for these instrumental imperfections are then applied to the actual data. For further details, see Forkey et al. (2005).

Data Analysis

- 13 Correct the separate intensity traces from Step 4 with the calibration factors determined in Step 12.
- 14 Temporarily combine the separate intensity traces from each molecule into one total intensity signal. Use that total to select molecules for further analysis.
Selected molecules should show a constant intensity followed by a single, discrete bleach-to-background change.
- 15 Subtract each background recorded after the bleach from each intensity trace.
- 16 Use the probe model described in the text (see the section entitled The Principles of polTIRFM in the article **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy [polTIRFM]** [Beausang et al. 2012c]) to solve for the set of $(\theta, \phi, \delta_s, \delta_f, \kappa)$ parameters that best fit the measured intensities. Repeat the analysis for all complete cycles before the bleach.
The theoretical dependence of intensity on $(\theta, \phi, \delta_s, \delta_f, \kappa)$ can be found in Forkey et al. (2005).
- 17 Repeat the parameter estimation for all successful molecules.
- 18 Interpret the data. Typically, $(\theta, \phi, \delta_s, \delta_f, \kappa)$ are not straightforward to interpret on their own and must be rotated into a frame of reference that is appropriate to the biological system. For details, see the section entitled The Principles of polTIRFM in the article **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM)** (Beausang et al. 2012c).
- 19 Write the manuscript.

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

- Beausang JF, Sun Y, Quinlan ME, Forkey JN, Goldman YE. The polarized total internal reflection fluorescence microscopy (polTIRFM) processive motility assay for myosin V. Cold Spring Harb Protoc. 2012a10.1101/pdb.prot069393
- Beausang JF, Sun Y, Quinlan ME, Forkey JN, Goldman YE. The polarized total internal reflection fluorescence microscopy (polTIRFM) twirling filament assay. Cold Spring Harb Protoc. 2012b 10.1101/pdb. prot069401.
- 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. 2012c10.1101/pdb.top069344
- Forkey JN, Quinlan ME, Goldman YE. Measurement of single macromolecule orientation by total internal reflection fluorescence polarization microscopy. Biophys J. 2005; 89:1261–1271. [PubMed: 15894632]

FIGURE 1.

The polTIRFM experimental apparatus. The input laser beam (green) is alternately directed along paths 1 and 2. For each path, the beam passes through a linear polarizer (P) and then a Pockels cell (PC) to generate alternating linear polarizations parallel (p) and perpendicular (s) to the relevant reflection plane. The beam is coupled into the quartz microscope slide by passing through the coupling prism (CP) and through index-matching liquid. At the quartz/water interface, total internal reflection occurs, sending the beam back out through the coupling prism to a beam dump (not shown). The quartz slide (*top left*) is mounted on a piezoelectric stage (PS). Fluorescence (red) is collected by a microscope objective lens (OL), passed through bandpass filters (BF), and imaged by lenses (L). Depending on the position of a removable mirror (RM), the fluorescence is imaged either onto an ICCD camera or through a Thompson beam-splitting prism (BSP) onto two avalanche photodiodes (APDs). (*Top left*) Orientation of the flow cell relative to the two paths.