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Construction of Flow Chambers 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 construct sample chambers (flow chambers) for polTIRFM motility assays. Each chamber can hold ~20 μ L of solution. To flow a solution through the chamber, the solution is added to the chamber with a pipette while wicking out the previous contents with filter paper. Each end of the coverslip should extend beyond the edge of the slide to support the pipette tip and filter paper. The flow rate can be roughly controlled by adjusting the contact area between the filter paper and the solution. The chambers can be used for investigating the motility of myosin V in vitro with the processive motility assay, as well as for assessing the motility of actin using the twirling assay.

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

Acetone

Ethanol (100%)

KOH (200 mM)

Poly(methyl methacrylate) (PMMA) (2 mg/mL; Sigma-Aldrich 370037), prepared in methylene chloride

Equipment

Coverslips, glass (24 $\times$ 40 $\times$ 0.17-mm thick; Fisher)

Plasma cleaner/sterilizer (Harrick Plasma PDC-32G)

Slides, fused silica (1-mm thick; Quartz Scientific)

Sonicator (Branson 3510)

Spin coater (Laurell WS-400A-6NPP/Lite)

Staining cuvette

Tape, double-sided adhesive (Scotch Double-Coated Permanent Tape)

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.

Clean the Slides

- 1 Immerse the slides in a staining cuvette with clean acetone.
- 2 Sonicate the slides for 5 min at 38–40°C.
- 3 Rinse the slides three times with H₂O.
- 4 Sonicate the slides for 20 min with 200 mM KOH.
- 5 Rinse the slides three times with H₂O.
- 6 Sonicate the slides for 5 min with clean (100%) ethanol.
- 7 Allow the slides to dry in a clean hood.

Construct the Flow Chamber

- 8 Immediately before performing the desired experiment, plasma-clean a slide (from Step 7) for 5 min.
*Experiments may include **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).*
- 9 Mount the slide in a spin coater, and spin at 5000 rpm. Drop 18 μ L of PMMA on the spinning slide, and wait 30 sec to create a uniform PMMA film that is ~2–3 nm thick.
The PMMA film scatters light weakly in a “dendritic” pattern.
- 10 Place a 6 $\times$ 30-mm strip of double-sided adhesive tape along each long edge of a cleaned coverslip, leaving an ~10-mm gap between them.
- 11 Adhere the coverslip at right angles to the center of the slide to make a flow chamber (Fig. 1 and Fig. 2).
- 12 To seal the edges, use a blunt object to press down on the slide over the region of the coverslip and tape.

RELATED INFORMATION

A detailed discussion of polTIRFM can be found in **Orientation and Rotational Motions of Single Molecules by Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM)** (Beausang et al. 2012c). Analysis of the data obtained in the processive motility assay for myosin V and the twirling filament assay is described in **The Acquisition and Analysis of Polarized Total Internal Reflection Fluorescence Microscopy (polTIRFM) Data** (Beausang et al. 2012d).

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FIGURE 1.

The polTIRFM experimental apparatus. The input laser beam (green) is alternately directed along path 1 and path 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 intensified charge-coupled device (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.

FIGURE 2.

A cross section of the polTIRFM setup at the sample, showing the glass coupling prism, the flow cell, and the objective lens of the microscope (not drawn to scale). The autofluorescence from the glass prism is generated outside the field of view and thus does not contribute significantly to the background intensity.