Protocol

Tracking Movements of the Microtubule Motors Kinesin and Dynein Using Total Internal Reflection Fluorescence Microscopy

Ahmet Yildiz and Ronald D. Vale

Total internal reflection fluorescence microscopy (TIRFM) is a wide-field illumination technique that illuminates only the molecules near the glass coverslip. It has become widely used in biological imaging because it has a significantly reduced background and high temporal resolution capability. The principles of TIRFM are illustrated in this protocol, in which the movements of motor proteins are imaged as they move along microtubules within live axonemes.

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 protocol for recipes indicated by <R>. Additional recipes can be found online at http://cshprotocols.cshlp.org/site/recipes.

Reagents

β-mercaptoethanol (BME)
Biotinylated motor protein (either dynein or kinesin)
BRB12 buffer (for the study of kinesin) <R>
Casein (blocking agent) solution prepared from lyophilized powder

Do not use dry milk because it contains massive amounts of biotin.

Dynein loading buffer (DLB) (for the study of dynein) <R>
Glucose (40%, w/v)
Mg^{2+}-ATP (100 mM)
Oxygen-scavenging agents (glucose oxidase (100 mg/mL) and catalase) (Yildiz et al. 2003)
Quantum dots, streptavidin-coated
Sea urchin axonemes

Equipment

Coverslip
Microscope slide
Nail polish
Tape, double-sided
TIRFM setup equipped with 488-nm laser, quantum dot filter set, and an EMCCD camera

Adapted from *Imaging: A Laboratory Manual* (ed. Yuste). CSHL Press, Cold Spring Harbor, NY, USA, 2011.

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot086355

METHOD

Sample Preparation

1. Prepare a flow chamber by sandwiching a slide and a clean coverslip with two pieces of double-sided tape. Solutions flow through the two open ends of the chamber, which holds ~10 µL of aqueous solution.
2. Flow 10 µL of sea urchin axonemes (1 mg/mL) into the chamber. Axonemes stick nonspecifically to the chamber surface and are stable for several hours at room temperature.
3. Remove unbound axonemes with 50 µL of either BLB12 or DLB buffer.
4. Block the surface with buffer containing 1 mg/mL casein (buffer + casein).
5. Add 40 µL of 100 pM biotinylated motor protein diluted in buffer + casein.
6. Remove unbound motor protein with two washes of 100 µL buffer + casein.
7. Add 10 µL of 1 µM quantum dots and incubate the sample for 5 min to allow streptavidin–biotin attachment.

   Labeling the microtubule-bound motors with quantum dots is performed to prevent cross-linking of multiple motors to single quantum dots. 1 mg/mL of casein is absolutely required to prevent the quantum dots from sticking to the chamber surface.
8. Remove excess quantum dots with two washes of 100 µL buffer + casein.
9. Add 20 µL of buffer + casein containing 1 mM ATP, 140 mM of BME, and 1 mg/mL casein and oxygen scavenging mixture (1 µL glucose oxidase, 1 µL catalase, and 1 mL glucose).

   BME enhances the brightness of the quantum dots by reducing the rates of fluorescent blinking (Rasnik et al. 2006).
10. Seal the sample with clean nail polish.

Image Acquisition and Data Analysis

11. Excite the samples with 20 mW of a 488-nm laser with TIRFM.
12. Find the coverslip surface by locating the back-reflected beam on TIRF lens next to the illumination spot and by observing the quantum dot signal through microscope binoculars.
13. Direct the fluorescence signal to the camera port.
14. Acquire images at a 10-MHz readout rate in a frame transfer mode. EM cameras with 128 × 128 pixels and 512 × 512 pixels can acquire 500 frames and 30 frames per second, respectively.
15. Use 60× EM gain to reduce the readout noise rate to below 1 electron rms.
16. Collect 2000–20,000 images per movie.
17. Use the program of choice (e.g., IDL, MATLAB, C + +) to find fluorescent spots with Gaussian masking and track the spots throughout the movie. Using a two-dimensional Gaussian fitting, determine the position of the quantum dots in each image.
18. Fit the resulting position versus time plot to a stepping algorithm to determine the sizes of the nanometer-range steps taken by the molecular motors.

RELATED INFORMATION

For an introduction to TIRFM, see **Total Internal Reflection Fluorescence Microscopy** (Yildiz and Vale 2015).

Cite this protocol as *Cold Spring Harb Protoc*; doi:10.1101/pdb.prot086355

RECIPES

BRB12 Buffer

PIPES (pH 6.8)	12 mM
EGTA	1 mM
$MgCl_2$	2 mM

Dynein Loading Buffer

HEPES (pH 7.2)	25 mM
EGTA	1 mM
$MgCl_2$	2 mM

REFERENCES

Rasnik I, McKinney SA, Ha T. 2006. Nonblinking and long-lasting single-molecule fluorescence imaging. *Nat Methods* **3:** 891–893.

Yildiz A, Vale RD. 2015. Total internal reflection fluorescence microscopy. *Cold Spring Harb Protoc* doi: 10.1101/pdb.top086348.

Yildiz A, Forkey JN, McKinney SA, Ha T, Goldman YE, Selvin PR. 2003. Myosin V walks hand-over-hand: Single fluorophore imaging with 1.5-nm localization. *Science* **300:** 2061–2065.