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Single-Molecule Gold-Nanoparticle Tracking

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Abstract

Gold nanoparticles, like single fluorophores, can be used to locate single molecules with nanometer accuracy. Unlike an optical trap, the gold particle label does not exert an external load, which is important for studying diffusive processes. Thus, a gold particle can be used analogously to a single fluorophore, providing similar information but with submillisecond time resolution. The features of gold-nanoparticle tracking (high temporal resolution, small label size, and lack of applied force) facilitate the characterization of structural properties of short-lived intermediates, as shown by our work with myosin V. This protocol provides details for gold-nanoparticle-tracking experiments, including flow cell construction, microscopy, and data analysis, along with a brief outline of actin and myosin preparation. Although details particular to our experiment are given, the approach should be generally applicable.

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.

RECIPE: 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

ABcam (5 μ M calmodulin in Assay buffer [pH 7.4])

Actin stock solution <R>

Assay buffer (pH 7.4) <R>

Biotin-maleimide (50 mM in DMSO; Sigma-Aldrich)

Aliquots can be frozen and stored at -80°C , but only thawed once.

Biotinylation buffer (5 mM CaCl_2 , 20 mM imidazole; pH 7.5)

Bovine serum albumin (BSA) (99%; Sigma-Aldrich)

CaCl_2 (10 mM)

Calmodulin

Express and purify sea urchin vertebrate-like calmodulin bearing the mutation Q143C as described previously (

Churchman et al. 2005).

Dialysis buffer (pH 7.5) <R>

EGTA (500 mM, pH 8.0)

Exchange buffer (pH 7.5) <R>

GO buffer (pH 7.4) <R>

Gold particles, 40-nm diameter, streptavidin-conjugated (Ted Pella)

Myosin

Express and purify a chicken myosin V/GCN4 fusion plus calmodulin as described previously (

Churchman et al. 2005).

Nitrocellulose (12620-10, Electron Microscopy Sciences), 0.1% in *n*-amyl acetate (>99%; W504009, Sigma-Aldrich)

Reagents for MALDI-mass spectroscopy, in addition to those listed

Surface passivation polymer (e.g., PLL(20)-g[3.5]-PEG(2)/PEG(3.4)-Biotin [this protocol] or PLL

(20)-g[3.5]-PEG(2); SurfaceSolutions, GmbH 1 mg/mL in PBS)

Tris (2 mM, pH 8.0)

Equipment

Air table (Newport Corporation)

Buffer exchange column (Micro Bio-Spin 6; BioRad)

Camera (Andor DV860 EMCCD [electron multiplying charge-coupled device])

Centrifuge

Condenser (reflecting ball, 1.2–1.33 numerical aperture [NA]; Olympus)

Coverslips (no. 1 weight)

Equipment for matrix-assisted laser desorption/ionization (MALDI)-mass spectroscopy, in addition to the items listed

Goggles, laser safety (532 nm)

Grid (10 μ m; Technical Instruments, Burlingame, CA)

Laser (Coherent DPSS, 532 nm, 100 mW)

This is a Class IIIB laser, which can instantaneously cause irreversible eye damage. Use appropriate laser goggles at all times.

Microscope

This should be upright, with standard visible-wavelength mirrors and mounts, and a water immersion, fixed-length objective (40 $\times$, 0.9 NA) (Zeiss).

Plasma Cleaner (Harrick Plasma)
 Slides (3050, Gold Seal)
 Tape (double-sided, transparent; Scotch)
 Water bath preset to 22°C

METHOD

Cysteine-specific Biotinylation of Calmodulin

- 1 Transfer the Q143C calmodulin into biotinylation buffer using a Micro Bio-Spin 6 buffer exchange column. Dilute to a final protein concentration of 6.7 mg/mL.
- 2 Add biotin-maleimide to a final concentration of 0.5 mM. Incubate for 1 h at 22°C.
- 3 Dialyze against dialysis buffer overnight at 4°C.
- 4 Check biotinylation by MALDI-mass spectroscopy.
- 5 Flash-freeze aliquots. Store at -80°C.

Attachment of Biotinylated Calmodulin to Myosin

Below is a slight modification of an established method to attach the biotinylated calmodulin to myosin V (Churchman et al. 2005).

- 6 Add wild-type and biotinylated calmodulin to exchange buffer to final concentrations of 0.75 μ M each.
- 7 Add myosin V (~70 nM heavy chain). Incubate for 2 min at 22°C.
- 8 Add CaCl₂ to 1 mM (final concentration) to initiate calmodulin exchange. Incubate for 5 min.
- 9 Quench by adding EGTA to 8 mM (final concentration).

Streptavidin-mediated Conjugation

- 10 Prepare the gold particle suspension as follows:
 - i. Dilute 30 μ L of gold nanoparticle-streptavidin conjugates to 1 mL with 2 mM Tris (pH 8.0).
 - ii. Centrifuge at 14,000g for 2 min.
 - iii. Resuspend the pellet in 30 μ L of 2 mM Tris (pH 8.0).
 - iv. Repeat twice (three washes total).
- 11 Dilute the gold particle suspension 1:1 with ABcam.
- 12 Add serial dilutions of biotinylated myosin V (prepared in Steps 6–9) to the gold particle suspension. (Final myosin V and particle concentrations are ~50 and 300 pM, respectively, consistent with one or zero myosin dimers per gold

particle.) Because of day-to-day variability, always test a range of myosin to gold-particle ratios, and use the lowest ratio that produces moving particles.

The myosin V–gold conjugates are stable for several hours at 4°C.

See Troubleshooting.

Flow Cell Assembly

Experiments performed in the presence of high ionic strength require an altered flow cell assembly, because the actin filaments release from the slide surface under these conditions. (See Step 14 procedure for high-ionic-strength applications.)

- 13 Treat glass microscope slides in the plasma cleaner for 1 min on “high” and a pressure of 2 Torr.
- 14 Use one of the following two methods for flow cell assembly.

For normal flow cell assembly

- i. Assemble a single flow cell using double-sided transparent tape and a coverslip (Rock et al. 2000).
- ii. Add 15 mL of a 1 mg/mL solution of biotinylated poly(ethylene glycol)-poly-L-lysine (PEG–PLL) branch copolymer in PBS to the flow cell. Incubate for 30 min (Valentine et al. 2006).

The polymer passivates the surface. In our particular experiment, it also provides weak interactions that immobilize actin. The biotinylation of the PEG–PLL is unlikely to be important in our assay.

- iii. Wash the cell twice with 15 µL of assay buffer.
- iv. Wash the cell four times with 10 µL of the actin stock solution diluted 1:20 in assay buffer.

Care must be taken to use gentle, gravity-driven flow to avoid dislodging the filaments.
- v. Wash twice with 15 µL of assay buffer to remove excess actin.
- vi. Dilute the myosin V–gold conjugate 1:3 in ABCam. Add 10 µL to the cell. Incubate for 10 min to allow rigor binding to actin.
- vii. Remove unbound particles with 15 µL of ABCam.

For high-ionic-strength applications

- i. Coat the slides by centrifugation at 4000 rpm with 0.1% nitrocellulose in *n*-amyl acetate for 40 sec.
- ii. Assemble a single flow cell using double-sided transparent tape and a coverslip.
- iii. Dilute the actin stock 1:20 in assay buffer. Apply 10 µL to the cell. Incubate for 2 min.

- iv. Wash the cell twice with 15 μ L of assay buffer to remove excess actin.
 - v. Add 15 μ L of surface passivation polymer (1 mg/mL solution of biotinylated PEG–PLL branch copolymer in PBS) to the flow cell. Incubate for 30 min.
 - vi. Add 15 μ L of 10 mg/mL BSA to further reduce particle sticking. Incubate for 2 min.
 - vii. Dilute the myosin V–gold conjugate 1:3 in ABcam containing 1 mg/mL BSA. Add 10 μ L to the cell. Incubate for 10 min to allow rigor binding to actin.
 - viii. Remove unbound particles with 15 μ L of ABcam containing 1 mg/mL BSA.
- 15 Add 10 μ L of GO buffer.

Microscopy

- 16 Examine the flow cell using dark-field illumination (Fig. 1), with a 100-mW 532-nm laser as illumination source. (A 1-OD neutral density filter reduces the illumination intensity $\sim$ 10 mW.) Focus and steer the laser through the flow cell using a 1.2–1.33 NA reflecting ball con denser. Collect scattered light with a 40 $\times$ 0.9 NA Zeiss water-immersion, fixed-length objective.
- During data collection, keep the sample at $\sim 20 \pm 2^\circ\text{C}$.
- See Troubleshooting.
- 17 Record the image with an Andor DV860 back-illuminated EMCCD camera, at frame rates up 3125 Hz.
- 18 Calibrate pixel size using a 10- μ m grid.
- The uncertainty in pixel size derived from this calibration is 0.35%, measured as the uncertainty of the mean distance between grid marks.

TROUBLESHOOTING

Problem (Step 12): Particles disappear or turn purple, indicating particle aggregation.

Solution: Naked gold particles are only soluble in very low-ionic-strength (i.e., <5 mM) solutions. Fortunately, gold particle–protein conjugates are soluble in common buffers. In our hands, store-bought conjugates are quite reliable. Homemade conjugates can be finicky. To avoid aggregation, work at lower ionic strength, include a passivating agent in the buffer (BSA works well), or buy new conjugated particles.

Problem (Step 16): Particles stick to the glass surface.

Solution: Modify the surface passivation used in Step 14. Several observations may be helpful in modifying these methods for use with other experimental systems:

1. We found the PEG–PLL copolymer to be highly effective in preventing nonspecific sticking. Its use is therefore encouraged.

2. In our hands, streptavidin-coated gold particles stick to surfaces coated with streptavidin, avidin, or neutravidin.
3. Other surface-passivating proteins, notably casein, have been found to be preferable to BSA in some applications.

Problem (Step 16): There is difficulty aligning illumination and collection paths.

Solution: Alignment can be a bit difficult when the instrument is first being built. Consider the following:

1. Imaging micron-size silica or polystyrene beads, which scatter very strongly, is quite helpful.
2. Finding the focal plane of the objective using bright-field or fluorescence imaging, and then walking the dark-field illumination beam into the fixed field of view can also help.

DISCUSSION

Background

Gold nanoparticles scatter light about 1000 times better than plastic beads of the same diameter (Yguerabide and Yguerabide 1998a). Under dark-field illumination, they appear as bright spots against a dark background, analogous to single fluorophores. As with well-known single-fluorophore techniques (e.g., Yildiz et al. 2003), the location of the gold particle can be determined with nanometer accuracy. Unlike fluorophores, the photon flux is not limited by the excited-state lifetime, bleaching, or blinking. It is therefore possible to track gold nanoparticles with submillisecond resolution (Yasuda et al. 2001; Dunn and Spudich 2007). Unlike an optical trap, the gold particle label does not exert an external load—a crucial property for studying diffusive processes. Thus, a gold particle can be used analogously to a single fluorophore, providing similar information but with better time resolution.

Our work (Dunn and Spudich 2007) builds on previous results (Schafer et al. 1991; Malik et al. 1994; Yasuda et al. 2001) and serves as a concrete example of the utility of gold-particle tracking. Myosin V moves cargoes along actin filaments by walking hand-over-hand (De La Cruz et al. 1999; Mehta et al. 1999; Veigel et al. 2002; Churchman et al. 2005; Sellers and Veigel 2006). Little is known about the fleeting intermediate that occurs when the rear head detaches from the filament. We used submillisecond dark-field imaging of gold-nanoparticle-labeled myosin V to observe directly the free head as it releases from the actin filament, diffuses forward, and rebinds (Fig. 1). The increase in position variance that we observe during the one-head-bound intermediate (Fig. 2) is consistent with the simple model that the unbound head rotates freely about the lever arm junction. This trait likely facilitates travel through crowded actin meshworks.

Analysis of Results

The light scattered by an individual gold particle using this protocol can be treated as a point source because the gold particles are significantly smaller than the diffraction limit on the

resolution of the microscope. The collected photons form an Airy disk, which we fit with a two-dimensional Gaussian. Raw data were imported into Matlab, and the spot center for each frame was determined using a double Gaussian fit (Thompson et al. 2002; Yildiz et al. 2003; Ökten et al. 2004; Churchman et al. 2005). An actin filament, modeled as a second-order polynomial, was then fit to the path of the particle. The data shown in Figure 1 (lower panel) correspond to the distance traversed along the filament.

We analyzed data collected for 40 immobile particles. All analyzed stuck particles were 40-nm gold particles, recorded at 3125 Hz. Windows of 144 msec for each particle were selected for localization. On average, the spot centers had x and y standard deviations of $\sigma_x = 11.6$ and $\sigma_y = 10.6$ nm, respectively, over the course of the window. The 10% of stuck particles with lowest noise had $\sigma_x = 6.8$ nm and $\sigma_y = 5.8$ nm. These figures likely represent the current noise limits of the instrument.

We were chiefly interested in large (i.e., >10 nm) motions. Thus, we have not gone to great lengths to exclude external noise. Marked improvements in spatial resolution should be possible with improved vibrational isolation and air-handling control, analogous to the approaches used in optical trap design (Abbondanzieri et al. 2005).

Strengths and Weaknesses of Gold-Particle Tracking

Localization accuracy with single-molecule tracking is dependent on the number of photons collected per frame. A rule of thumb is that 104 photons per frame are sufficient for nanometer localization (Thompson et al. 2002; Yildiz et al. 2003). This relatively large number of photons constrains the maximal temporal resolution available using spot-localization approaches. Neglecting other factors, a typical fluorophore with an excited-state lifetime of 1 nsec and a microscope with 1% photon detection efficiency, would yield a maximum frame rate of 1000 Hz. However, this calculation ignores fluorophore bleaching or blinking, as well as background noise, all potentially serious experimental difficulties. Although quantum dots show promise, fluorescent labels currently lack the photon flux necessary for submillisecond localization.

Gold-particle tracking offers submillisecond temporal resolution and nanometer resolution without the application of an external load. Gold particles have additional practical benefits as single-molecule labels. Their scattering intensity peaks at 532 nm (Yguerabide and Yguerabide 1998b), which is well matched to common frequency-doubled Nd:YAG lasers. Gold particles conjugated to streptavidin, protein A, and various antibodies are commercially available (Ted Pella). Because gold particles are useful for electron microscopy labeling, diagnostics, and other applications, a large body of literature exists on their handling and derivatization (Schultz et al. 2000; Parak et al. 2003; Daniel and Astruc 2004; Doty et al. 2004; Sonnichsen et al. 2005).

Like any technique, gold-nanoparticle dark-field imaging has drawbacks. There is no appreciable Stokes' shift between the incident and scattered light, which means that the excitation light cannot be easily removed with filters. Extraneous dirt and surface imperfections can scatter light appreciably, leading to background problems. Gold particles, at approximately 40 nm in diameter, are somewhat larger than quantum dots (~20 nm

diameter) and much larger than single fluorophores (~1 nm). Finally, single-molecule fluorescence experiments often implicitly rely on photobleaching to remove uninteresting fluorophores from the field of view. Because gold particles do not photobleach, stuck particles remain in view indefinitely.

Theoretical Considerations

A few simple equations should be considered when designing a single-molecule gold-nanoparticle experiment. The Rayleigh equation for the scattered light intensity I for a given particle is:

$$I \propto \frac{a^6}{\lambda^4} \left| \frac{(m/n)^2 - 1}{(m/n)^2 + 2} \right|^2$$

Here, a is the particle radius, λ is the illumination wavelength, m is the particle index of refraction, and n is the medium index of refraction (1.33 for water; Yguerabide and Yguerabide 1998a). Gold has a complex m , with a value of roughly $0.3 + 2i$ at 530 nm, whereas m is 1.6 for polystyrene. This difference in material properties means that gold particles scatter light about 1000 times more effectively than polystyrene particles of the same radius. However, scattering also depends on a^6 . This means that a 40-nm gold particle scatters light about as well as a 125-nm polystyrene bead.

This factor of three in radius can decisively affect the success of the experiment. Smaller labels are desirable where steric hindrance is a concern, as in our experiment. The time resolution of the measurement also improves with smaller particles. The relaxation time of a particle in a linear potential is:

$$\tau = \frac{\gamma}{\kappa} = \frac{6\pi\eta a}{\kappa}$$

where γ is the drag coefficient, η is the medium viscosity, a is particle radius, and κ is the stiffness of the restorative force due to the bead–myosin attachment. If $\kappa = 1$ pN/nm, τ is 0.3 μ sec, which is very fast compared to current measurements. However, for a bead rotating about its edge in a torsional potential, the relaxation time is (Yasuda et al. 1996):

$$\tau_{\text{rot}} = \frac{\gamma}{\kappa} = \frac{14\pi a^3 \eta}{\kappa}$$

using $\kappa = 1$ pN nm, $\tau_{\text{rot}} = 0.4$ and 12 msec for 40- and 125-nm diameter particles, respectively. Here, the time resolution of the experiment is markedly improved by using the smallest possible particle. Note also that these crude calculations suggest that the particle relaxation will be dominated by rotational degrees of freedom.

Instrumentation

Published experiments use fast cameras (Dunn and Spudich 2007) for tracking. Complementary metal oxide semiconductor (CMOS) cameras have excellent temporal resolution (~10 kHz) and pixel numbers. However, their quantum efficiencies are low. In contrast, EMCCD cameras have very high detection efficiencies, but maximum frame rates are about 3 kHz (Yasuda et al. 2001). Both cameras have been used successfully. A possible drawback to CMOS cameras is that the necessary illumination intensity (~200 mW) could in principle result in a modest (0.01–0.1 pN) force exerted on the particle. This load would have little effect on most measurements but could perturb diffusive processes. We used a 100-mW laser, but discarded most of the available power. Similar measurements could be accomplished with less expensive, and safer, 10–25 mW lasers.

Currently published gold-particle-tracking experiments as of this writing have single-millisecond or slightly better time resolution. Many interesting processes occur on the 10–100 μ sec timescale. Tracking individual particles with an avalanche quadrant photodiode or a position-sensitive device in principle offers much better temporal resolution. On these timescales, the particle relaxation time will become important. Stiff connections to the molecule of interest and smaller particles will be necessary.

To our knowledge, currently published gold-particle-tracking measurements as of this writing use wide-field illumination techniques. In principle, total internal reflection (TIR) illumination should reduce the background caused by out-of-focus spots. In addition, TIR illumination should allow higher particle concentrations, which would improve experimental throughput. TIR-based dark-field illumination geometries have been described that could in principle be used for gold-particle-tracking experiments (Braslavsky et al. 2001; Zocchi 2001; Dornier et al. 2004).

RECIPES

Actin Stock Solution

10 μ M actin, prepared as described previously (Pardee and Spudich 1982) but using 1 mM dithiothreitol instead of β -mercaptoethanol

1 mM ATP

1 mM DTT

6.7 μ M phalloidin

3.3 μ M tetramethylrhodamine phalloidin (rhodamine phalloidin; Invitrogen)

Prepare in an assay buffer (pH 7.4). The actin stock solution can be kept for ~1 mo at 4°C.

Assay Buffer (pH 7.4)

25 mM imidazole

25 mM KCl

4 mM MgCl₂

1 mM EGTA

Dialysis Buffer (pH 7.5)

0.5 mM DTT

0.5 mM EGTA

10 mM imidazole

Exchange Buffer (pH 7.5)

25 mM imidazole

25 mM KCl

4 mM MgCl₂

GO Buffer (pH 7.4)

3 μM ATP

100 nM biotin

0.04 mg/mL catalase

0.1 mg/mL creatine phosphokinase

0.4% w/v glucose

0.2 mg/mL glucose oxidase

1 mM phosphocreatine

Prepare in assay buffer (pH 7.4) containing 5 μM calmodulin.

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FIGURE 1.

Tracking the motion of the myosin V lever arm with millisecond resolution. (*Top panel*) A myosin V dimer is labeled with a 40-nm-diameter gold particle (Au) on one of its lever arms through a biotin–streptavidin linkage. The myosin V–gold conjugate walks along surface-immobilized actin. Light scattered by the gold particle is collected by the objective and imaged at 3125 frames/sec. (*Middle panel*) The location of the gold particle is determined for each frame. Myosin walks left to right, resulting in a series of discrete steps. (*Lower panel*) Sample data trace; 40-nm-diameter gold particle, 3 μ M ATP. Frames were taken every 0.32 msec (blue); a 16-msec sliding average is shown in red. The step size (74 nm) and frequency match previously reported values (De La Cruz et al. 1999; Yildiz et al. 2003).

FIGURE 2.

Sample myosin V substeps recorded at 3125 Hz, using 40-nm gold particles. The displacement along the actin filament is shown in blue. The corresponding perpendicular displacement is shown immediately below in green. The end of the substep is indicated by an arrow. Note the increased variance in both directions during the intermediate. Transient lateral offsets are observed to both the *left* (positive) and *right*. The observed increase in variance is consistent with free rotation of the unbound head about the lever arm junction (Dunn and Spudich 2007).