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Coverslip Cleaning and Functionalization for Total Internal Reflection Fluorescence Microscopy

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Abstract

Total internal reflection fluorescence (TIRF) microscopy allows visualization of biological events at the single molecule level by restricting excitation to a precise focal plane near the coverslip and eliminating out-of-focus fluorescence. The quality of TIRF imaging relies on a high signal-to-noise ratio and therefore it is imperative to prevent adherence of molecules to the glass coverslip. Nonspecific interactions can make it difficult to distinguish true binding events and may also interfere with accurate quantification of background noise. In addition, nonspecific binding of the fluorescently tagged protein will lower the effective working concentration, thereby altering values used to calculate affinity constants. To prevent spurious interactions, we thoroughly clean the surface of the coverslip and then functionalize the glass either by applying a layer of silane or by coating with a lipid bilayer.

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 material used in this protocol.

Reagents

Biotinylated antibody (200 μ M stock solution)
BRB80 <R> (or any buffer with pH 5–9 and less than 200 mM ionic strength)
Butylamine
 κ -Casein (5 mg/mL stock solution; filter sterilized)
Chloroform
Concentrated HCl (37%)
Concentrated sulfuric acid (95–98%)
1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC; 10 mg/mL in chloroform)
(Avanti Polar Lipids)

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Ethanol
Hydrogen peroxide (30%)
Ice
Imaging buffer <R>
Methanol
2-Methoxy(polyethyleneoxy)propyltrimethoxysilane (Gelest SIM-6492.7)
Nitrogen gas (dry)
1-Oleoyl-2-(12-biotinyl(aminododecanoyl))-*sn*-glycero-3-phosphoethanolamine (Biotinyl Cap PE; 0.1 mg/mL in 2:1 chloroform/methanol) (Avanti Polar Lipids 870277X)
Purified protein of interest
Streptavidin (1 mg/mL stock solution)
Toluene
Wash buffer (BRB80 <R> containing 1 mg/mL κ -casein)

Equipment

Beaker (2 L with Teflon lid and gas inlets)
Beakers (glass; 1 L and 2 L)
Coverslips (glass; 22 × 60 mm)
Double-sided tape
Drying oven (set at 50°C)
Fume hood
Hot plate (set at 50°C)
Mercury lamp
Metal bar to hold Teflon racks
Nail polish
pH strips (range 2.0–7.0)
Pipettes (glass)
Plasma cleaner
Quartz cuvette
Razor blade
Safety gear (laboratory coat, goggles, gloves, and UV eye protection)
Slides (glass; 3 × 1 in)

Sonicator water bath
Sonifier (with microtip probe)
Spray bottle
Syringes (glass; 10 μ L and 100 μ L)
Teflon racks (custom-fabricated; see Fig. 1) Teflon straw
Test tubes (glass)
Vacuum pump and desiccator
Vials (glass; for storing lipids)

Use Teflon tape for sealing the vials. Do not use rubber cap liners.

Vortexer
Water bath (set at 50°C)

Method

Coverslips are thoroughly cleaned and the glass surface is functionalized either by applying a layer of silane (Method I; Steps 1–21) or by coating with a lipid bilayer (Method II; Steps 22–42).

I. Functionalizing Glass Coverslips by Silanization

The silanization method (adapted from Cras et al. 1999 and Walba et al. 2004) involves a harsh acid wash to clean the glass followed by application of a functionalized layer of silane, which adheres to the glass and creates a uniform, covalently linked layer on the coverslip surface. The silane is linked to a short polyethyleneglycol molecule at one end, creating a hydrophilic layer pointing away from the surface. The hydrophilic layer attracts water and helps prevent proteins from adhering to the coverslip.

Acid-Washing the Coverslips

1. Load glass coverslips (22 $\times$ 60 mm) into four custom-fabricated Teflon racks (Fig. 1).

A typical rack contains 13 slots and four units can be utilized together to clean and silanize 52 coverslips in one preparation. The racks are handled by inserting a horizontal metal bar into the top loops of each rack. All subsequent manipulations are performed with the coverslips loaded into the racks.

- 2 Holding the racks by the metal bar, briefly rinse the coverslips by dipping the racks into a 1-L beaker filled with distilled water.
- 3 Mix 500 mL of methanol with 500 mL of concentrated HCl in a 2-L beaker in the fume hood. Cool on ice for about 10 min (the reaction is exothermic) to allow time for the fuming to stop. Place the methanol/HCl beaker into a sonicating water bath.

- 4 Remove the coverslips from the distilled water and gently shake off excess water.
- 5 Carefully lower the coverslips into the methanol/HCl solution and gently dip up and down several times. Sonicate for 30 min in the sonicating water bath.
- 6 Prepare five wash beakers by filling five 2-L beakers with 1 L of distilled water each.
- 7 After sonication, gently shake off excess methanol/HCl solution and dip the coverslips several times into the first wash beaker. Repeat with the second wash beaker.
- 8 Place the second wash beaker into the sonicating water bath and sonicate for 3 min.
- 9 Thoroughly rinse the coverslips sequentially in the remaining wash beakers. Carefully dip the racks up and down several times in each wash solution. Check the pH of the water in each wash beaker using pH strips to ensure that the HCl is being washed away. (By the 4th or 5th beaker, the pH should equal to that of distilled water.) Thoroughly, but gently, shake off excess water after the final wash.
- 10 Pour 1 L of concentrated sulfuric acid into a clean 2-L beaker in the fume hood. Place the beaker into the sonicating water bath.
- 11 Lower the coverslips into the sulfuric acid, gently dipping up and down several times as in Step 5. Sonicate for 30 min.
- 12 Prepare seven wash beakers by filling seven 2-L beakers with 1 L of distilled water each.

These additional washes are required to fully rinse off the sulfuric acid.

- 13 Rinse the coverslips sequentially in the wash beakers and check the pH of the rinse water. Sonicate the wash beakers for 10 min during the second and third washes. (By the seventh wash, the sulfuric acid should be removed.) Shake off excess water following the final wash.
- 14 Fill a spray bottle with 300 mL of ethanol. Spray the coverslips extensively with ethanol and place them in the drying oven at 50°C for 30 min.
- 15 When the coverslips are thoroughly dry, continue with silanization (Steps 16–21).

Silanization

- 16 Mix the silanization solution in a 2-L beaker by combining 960 mL of toluene, 16.8 mL of 2-methoxy(polyethyleneoxy)propyltrimethoxysilane, and 6 mL of butylamine.

- 17 Transfer the acid-cleaned, dry coverslips into the silanization solution and cap with a Teflon lid with N₂ gas inlets. Incubate for 90 min under N₂ gas at a flow rate of 2–3 mL/min.
- 18 Prepare two wash beakers by filling two 1-L beakers with 500 mL of toluene each. After incubating for 90-min, remove the coverslips from the silanization solution and gently shake to remove excess liquid. Dip the coverslips sequentially into the toluene washes, thoroughly shaking off the liquid between each wash.
- 19 Place the coverslips into a clean 2-L beaker with a Teflon lid and gas inlets. Fit two Teflon straws through the gas inlets and cover the beaker with the lid. The straws should reach the bottom of the beaker to achieve the best results. Dry thoroughly with N₂ gas at a flow rate of 5 mL/min for 90 min.
- 20 Cure overnight with N₂ gas at a flow rate of 1 mL/min.
- 21 Turn off the N₂ gas the following morning and cap the inlets. Store the coverslips at room temperature under N₂. To use silanized coverslips for TIRF microscopy experiments, see Protocol: Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy<prot085563> (Kudalkar et al. 2015).

II. Functionalizing Glass Coverslips by Lipid Passivation

Besides silanization, coating surfaces with a lipid bilayer provides an alternative approach for specifically immobilizing molecules of interest, while eliminating nonspecific interactions between the surface and background molecules. In general, lipid bilayers appear to be inert to most biomolecules, including nucleic acids and most soluble proteins. For the purpose of TIRF imaging, lipid bilayers can be created by the spontaneous deposition of small unilamellar vesicles (SUVs) on a flat hydrophilic surface such as a clean glass coverslip (Sackmann 1996; Cremer and Boxer 1999). Lipids that carry modified head groups can be mixed with regular phospholipids to introduce specific immobilization of the molecule of interest on the surface. We use biotinylated phosphoethanolamine (bio-PE) as the affinity anchor. The density of the active linkers can be conveniently adjusted by altering the fractions of modified and regular lipids. In addition, the extensive variety of lipid species allows one to tune the physical properties of the lipid bilayer such as its diffusivity and phase.

Preparation of Small Unilamellar Lipid Vesicles

- 22 In the fume hood, transfer 1 mL of chloroform into three glass test tubes for washing the glass syringes.
- 23 Wash a fourth test tube briefly with chloroform and drain. This tube will be the dry lipid test tube.
- 24 Wash a 100-μL glass syringe three times with the washing chloroform in each of the three test tubes before use. Transfer 70 μL of chloroform to the lipid test tube.

- 25 Transfer 30 μL of the DPPC lipid solution to the lipid test tube. Wash the 100- μL glass syringe nine times with the washing chloroform.
- 26 Wash the 10- μL glass syringe nine times with the washing chloroform. Transfer 4 μL of bio-PE lipid to the lipid test tube. Wash the 10- μL syringe nine times.
- 27 Vortex to mix the lipid.
- 28 Evaporate the chloroform from the mixture in the lipid test tube by gently blowing N_2 gas above the mixture for at least 5 min while slowly rotating the tube.

The dried lipid mixture will form a white patch at the bottom of the test tube.

- 29 Place the lipid test tube in the desiccator and further dry under vacuum for at least 15 min.

A plasma cleaner can also be used to dry the lipid. Keep the plasma off if drying with plasma cleaner.

- 30 Add 200 μL of BRB80 (or other desired buffer) to the dried lipid and vortex briefly. Place the test tube in a water bath set at 50°C . Vortex every 5 min until the lipid patch at the bottom of the tube is completely rehydrated into giant unilamellar vesicles (GUVs), which appear as a white opaque suspension.

This step typically takes less than 15 min.

- 31 Transfer the rehydrated lipid to a 1.5-mL microcentrifuge tube. Immerse the sonifier microtip probe into the suspension and sonicate for 3 min, while keeping the test tube in a water bath set above 50°C . To prevent spurious bubbling, lower the sonication duty cycle and the output power.

The white GUV suspension should turn into a clear SUV suspension.

- 32 (*Optional*) If low fluorescence background is required, add 100 μL of warm 30% hydrogen peroxide to the liposome suspension and transfer the mixture to a quartz cuvette. Place the cuvette under a mercury lamp and irradiate for 30 min to bleach the fluorophores in the liposome.

This step can be performed at room temperature. Proper eye protection is required to prevent UV damage.

UV irradiation and peroxide treatment have no observable effect on the passivation ability of saturated phospholipid and the biotin functional group, but they significantly lower the fluorescence of the contaminants in the liposome.

- 33 Keep the SUV preparation on a hotplate or in a water bath set at 50°C . Use the liposomes within 1 d.

Coating Glass Coverslips with a Lipid Bilayer

- 34 Clean coverslips and glass slides with a plasma cleaner.

- 35 Apply several pieces of double-sided tape crosswise to a plasma-cleaned glass slide, leaving ~1–3 mm gaps in between the pieces to create chambers.
- 36 Place a plasma-cleaned coverslip on the slide. Gently press down to ensure a tight and even seal between the coverslip and the tape. Place on a hot plate set at 50°C for 5 min and periodically press down on the coverslip to secure sealing.
- 37 Using a razor blade, trim off excess tape from either side of the coverslip so it is flush with the edge.
- 38 Transfer the slide to a warm humidity chamber, such as a pipette tip box half-filled with hot water. Flow in BRB80 and pre-wet the chamber to prevent lifting of the double-sided tape. Flow through SUV in warm buffer (from Step 33). Incubate for 5 min to allow the lipid bilayer to form. Prior to or during the incubation, prepare a 0.25 mg/mL solution of streptavidin in wash buffer.
- 39 Wash the chamber with 70 μ L of wash buffer. Move the slide to the humid chamber at room temperature. Flow through the streptavidin solution and incubate for 5 min. Prior to or during the incubation, prepare a 20 μ M solution of biotinylated antibody solution in wash buffer.
- 40 Wash the chamber with 70 μ L of wash buffer. Flow through the antibody solution. Incubate for 5–15 min. During the incubation, prepare the protein solution in wash buffer.
- 41 Wash the chamber with 70 μ L of wash buffer. Flow through the purified protein solution and incubate for 5 min. Prior to or during the incubation, prepare the imaging buffer.
- 42 Wash the chamber with 70 μ L of wash buffer. Flow through the imaging buffer, seal the chamber with nail polish. Observe the reaction under the microscope (see Protocol: Data Analysis for Total Internal Reflection Fluorescence Microscopy<prot085571> [Asbury 2015]).

Discussion

As with silanization, the lipid coating method provides a way to block nonspecific interactions between the glass surface of the coverslip and the solute while keeping a particular molecule of interest attached to the surface. We find the lipid coating procedure to be effective against nonspecific adsorption of DNA oligonucleotides, single protein molecules such as green fluorescent protein (GFP), and large protein complexes such as purified kinetochore particles. However, we do not recommend the lipid coating method when using microtubules because the lipid bilayer has an extremely high affinity for microtubules. (See Protocol: Preparation of Reactions for Imaging with Total Internal Reflection Fluorescence Microscopy<prot085563> [Kudalkar et al. 2015], for further details on using silanized coverslips in TIRF microscopy experiments for studying the binding of kinetochore proteins to microtubules.) Fine-tuning the ratio between functionalized and regular lipids can accurately control the density of the active functional groups on the bilayer surface. Each DPPC lipid occupies 50 $\text{\AA}^2$ and 0.1% of the lipid carries a biotin group

according to the ratio used in the protocol. Thus, the density of biotin on the bilayer is 2000 μm^{-2} .

Lipid bilayers have complex phase behavior according to temperature and composition. Above the transition temperature, the bilayer forms a two-dimensional fluid and the lipid molecules are diffusive. For TIRF imaging applications, the diffusion will cause motion blur, and the severity of this depends on the exposure time. This can be avoided by using lipids with high phase-transition temperature (such as DPPC) that can form a two-dimensional gel on the surface. However, the passivation efficiency is impaired if the lipid is in the gel phase. Supplementing the incubation buffer with κ -casein restores the passivation, presumably by blocking flaws in the gel-phase bilayer. When lateral constraint is not required, we recommend using low transition temperature lipids such as κ -POPC. In the latter case, all procedures can be performed at room temperature without κ -casein. The passivation efficiency of POPC is higher, and the POPC liposomes can be stored at 4°C for 3–5 d.

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Figure 1. Custom-fabricated Teflon coverslip rack. Our coverslip racks hold 13 glass coverslips (22×60 mm) with minimal contact and four units can be linked together to clean and silanize 52 coverslips in one preparation. Teflon can withstand all the chemicals used in the protocol and the racks can be reused for several years.