Please cite this article as: Zhengjian *et. al.*, (2016). Cloud-point PEG Glass Surfaces for Imaging of Immobilized Single Molecules by Total-internal-reflection Microscopy, *Bio-protocol* 6 (7): e1784. DOI: 10.21769/BioProtoc.1784.

bio-protocol http://www.bio-protocol.org/e1784 Vol 6, Iss 7, Apr 05, 2016

Cloud-point PEG Glass Surfaces for Imaging of Immobilized Single Molecules by Total-internal-reflection Microscopy

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[Abstract] This effective, robust protocol generates glass coverslips coated with biotin-functionalized polyethylene glycol (PEG), making the glass surface resistant to non-specific absorption of biomolecules, and permitting immobilization of biomolecules for subsequent single-molecule tracking of biochemical reactions. The protocol can be completed in one day, and the coverslips can be stored for at least 1 month. We have confirmed that the PEG surfaces prepared according to the protocol are resistant to non-specific adsorption by a wide range of biomolecules (bacterial, mitochondrial, and human transcription factors, DNA, and RNA) and biological buffers.

[Introduction] Single-molecule imaging methods of studying dynamics of biomolecules complement traditional 'bulk' biochemical methods by allowing real-time tracking of multi-step reactions without the need to synchronize the reagents (Weiss, 1999). In most single-molecule imaging methods, a biomolecule of interest is first labeled with a single fluorophore, the labeled biomolecule is then immobilized on an optically transparent surface (usually glass or silica), and detected as diffraction-limited image ('spot') using an optical microscope equipped with a high-sensitivity camera (Selvin and Ha, 2008). The surface immobilization serves two purposes. First, it permits tracking of molecular states on time scales longer than hundreds of milliseconds (otherwise, the biomolecule would diffuse out of the focal plane). Second, the surface permits excitation of fluorescence in total-internal-reflection geometry (Axelrod, 1981), which dramatically increases the signal-to-noise ratio of detection of molecules located in close proximity (<100 nm) to the surface (Selvin and Ha, 2008). Despite these clear advantages, surfaces are also the most common source of artifacts in single-molecule analysis (Visnapuu *et al.*, 2008). For example, irreversible, non-specific adsorption of bio-molecules onto the imaging surface may reduce the effective concentration of the bio-molecules in the 'bulk', and thus perturb the rate of the biochemical reaction. Moreover, if the 'sticky' molecule is fluorescently labeled, the noise from numerous non-specifically 'stuck' fluorescent molecules may obscure the signal from specifically bound molecules, which will complicate data analysis. Finally, tethering a biomolecule to a surface greatly increases the effective concentration of that molecule with respect to the surface, and further increases the probability of non-specific

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binding due to repetitive 'bumping' of the tethered molecule during long-term tracking. Overall, the compatibility of a surface for the biomolecules of interest needs to be validated on a case by case basis, and there remains a great demand in the single-molecule imaging field for effective, robust methods of surface passivation.

The current protocol builds upon a technique first introduced by Ha and colleagues (Ha *et al.*, 2002) which, in turn, built upon finding that polyethylene glycol (PEG) is most effective in creating anti-fouling surfaces (Prime and Whitesides, 1993), also see references in (Ostuni *et al.*, 2001). In the original protocol by Ha *et al.* (2002), glass surfaces were first coated with a silanol-reactive aminopropyltriethoxysilane (APTES) to create amine groups, followed by deposition of a mixture of amine-reactive N-hydroxysuccinimide (NHS)-PEG (to create a passivation layer on the glass) and NHS-PEG-biotin (to create a handle for immobilization of bio-molecules for single-molecule tracking). In our protocol, PEG deposition is performed in cloud-point conditions, which reduces the size of the PEG globule and results in a denser, more adsorption-resistant, PEG layer (Kingshott *et al.*, 2002). In addition, our protocol maximizes the reactivity of NHS-PEG during deposition. Furthermore, the protocol includes an end-capping step intended to eliminate residual amine groups remaining after PEG coupling, which we found to reduce non-specific adsorption of nucleic acids to surfaces in low-ionic-strength buffers required by some enzymes (Zhang *et al.*, 2014). Finally, the protocol provides simple quality-control tips to help trouble-shooting. Despite these key improvements, we found that some proteins are still prone to non-specific adsorption to 'cloud point' PEG surfaces. For instance, we found that the general transcription factor TFIID, a key component of the human transcription machinery, absorbs to 'cloud-point' PEG surfaces, whereas other five components of the basal human transcription machinery (TFIIB, TFIIF, TFIIE, TFIIH and RNA polymerase II) do not (Revyakin *et al.*, 2012). Thus, we recommend testing the 'cloud-point' PEG surfaces using your specific buffers, biomolecules of interest, and biochemical activity assays.

Materials and Reagents

1. Corning borosilicate cover glasses (24 x 40 mm, #1.5) (VWR International, catalog number: 48393-230)
2. PYREX crystallization dish (Thermo Fisher Scientific, catalog number: 08-741D)
3. Nalgene 125 ml polypropylene jars (Thermo Fisher Scientific, catalog number: 11-815-10C)
4. Two non-coring Syringe needles, luer, No 18, 6" long (Sigma-Aldrich, catalog number: Z102717-1EA)
5. Parafilm (100 mm wide) (various suppliers)
6. pH paper strips (0-14 range) (various suppliers)
7. Double-sided tape (3M formulation 4095) (McMaster, catalog number: 76665A67)
8. 30% hydrogen peroxide (Thermo Fisher Scientific, catalog number: H325-4)
9. 95-98% sulfuric acid (Sigma-Aldrich, catalog number: 320501-2.5 L)

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10. 99% 3-aminopropyltriethoxysilane (APTES) (Acros, catalog number: 430941000)
    Note: Stored under nitrogen in a container equipped with a rubber septum.
11. Acetone (Chromasolv for HPLC) (Sigma-Aldrich, catalog number: 270725-1 L)
12. Biotin-PEG-succinimidyl valerate [(bio-PEG-SVA) M_w = 5,000], (Laysan Bio)
    Note: Aliquoted into individual microtubes (1-2 mg/tube) by the end users and stored under desiccation at -80 °C.
13. Methoxy-PEG-succinimidyl valerate [(mPEG-SVA), M_W= 5,000] (Laysan Bio)
    Note: Aliquoted into individual microtubes (~5 mg/ tube) by the end users and stored under desiccation at -80 °C, with the mass of reagent written for each aliquot with 0.1 mg accuracy (e.g., 4.9 mg, 5.1 mg, and so on).
14. Fluorescein-PEG-NHS (Nektar, catalog number: 1K4M0F02)
15. Sulfosuccinimidyl acetate (Thermo Fisher Scientific, Pierce™, catalog number: 26777)
16. TRIONE ninhydrin reagent (Pickering Laboratories, catalog number: T100)
17. Potassium hydroxide (KOH) (semiconductor grade) (Sigma-Aldrich, catalog number: 306568-100 G)
18. Sodium bicarbonate ($NaHCO_3$) (ACS grade) (Sigma-Aldrich, catalog number: S6014-25 G)
19. Potassium sulfate (K_2SO_4) (ACS grade) (Sigma-Aldrich, catalog number: 221325-500 G)
20. Protein samples labeled at > 70% with a fluorophore suitable for single-molecule imaging with 532 nm or 640 nm excitation (*e.g.,* Cy3, Alexa555, Atto633 and Alexa647N)
    Note: These should be the protein of interest to the end user. Commercially available ones can also be used.
21. 0.5 M KOH (see Recipes)
22. 0.5 M K_2SO_4 (see Recipes)
23. 1 M $NaHCO_3$ (see Recipes)
24. Phosphate-buffered saline containing 0.1% Tween-20 (see Recipes)

Equipment

1. Ceramic rack (for 12 coverslips) (Thomas Scientific, catalog number: 8542E40)
2. Forceps (for handling of ceramic staining racks) (Thermo Fisher Scientific, catalog number: 10-316C)
3. Flat-tip tweezers (for handling of individual cover glasses) (Electron Microscopy Sciences, catalog number: 78335-35A)
4. 250 ml PYREX beaker (various suppliers)
5. A compressed nitrogen cylinder with a regulator and 0.2 micro filter (various suppliers)
6. Two-Way Valve (PTFE) (Sigma-Aldrich, catalog number: 20926)
7. UV-Vis spectrophotometer (various suppliers)

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8. Horizontal platform shaker (common lab equipment) (various suppliers)
9. Bath sonicator (Sonicsonline, Branson, catalog number: 2510)
10. Ultrapure water system (Merck Millipore Corporation)
11. Diamond scriber (Ted Pella, catalog number: 54463)
12. Objective-type total-internal-reflection (TIR) microscope (Olympus TIRF objective lens NA = 1.49) equipped with an electron-multiplication CCD camera (Andor iXon+) and 532 and 640 nm laser excitation sources (various suppliers)

Procedure

A. Piranha preparation and treatment to clean cover glasses

Note: Be very careful as the Piranha solution used in this step is extremely corrosive. Follow your institution's safety regulations. We recommend, at least, wearing a lab coat, a full-length rubber apron coat, long-sleeve butyl gloves, a full-face splash shield, and working under a chemical fume hood free of flammable organic chemicals. Use PYREX glassware for preparation of Piranha solutions (regular soda-lime glass beakers may crack upon heating when Pirahna solutions are prepared).

1. Place 12 coverslips into ceramic rack using flat-tip tweezers.
2. In a designated chemical fume hood, place two clean 250 ml PYREX beakers (beaker 1 and 2) in two separate crystallization dishes. The dishes serve as secondary containers to ensure safety.
3. Very carefully add 1 part (50 ml) of 30% hydrogen peroxide (H_2O_2) to beaker 1, followed by 3 parts (150ml) of concentrated sulfuric acid (H_2SO_4). Gently stir to mix the solution with a clean glass rod. The solution will immediately form bubbles and heat up to ~100 °C.
4. Using forceps, very carefully transfer the rack with the coverslips into beaker 1 and incubate for 30 min. To ensure safety, leave a note indicating that Piranha is in use.
5. Repeat Piranha treatment one more time with a fresh Piranha solution in beaker 2.
6. Transfer the coverslips from beaker 2 into a Nalgene 125 ml polypropylene jar filled with double-distilled water. Dispose of piranha waste according to your institution's regulations. Rinse the coverslips copiously with double-distilled water until pH stabilizes (verified by pH paper). The cleaned coverslips can be stored in double-distilled water without noticeable changes in reactivity and fluorescent background for at least 1 month.
7. Quality control.
   a. Surface hydrophilicity. Piranha-treated coverslips become uniformly hydrophilic, which can be qualitatively verified by dipping a coverslip in water using flat-end tweezers, taking it out vertically, and observing water slowly receding as a uniform sheet, and forming Young's rings before drying out. In contrast, untreated coverslips form patches of water when dipped into and taken out of water

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b. Fluorescent background. After Piranha treatment, blow-dry a coverslip with pressurized pure nitrogen (see step C12) and place it onto a total-internal-reflection microscope. One should observe essentially no fluorescent spots (<3 identifiable 'spots' per 100 x 100 micron area) under typical single-molecule imaging conditions (excitation with 532 and 640 nm sources at density ~0.5 kW/cm^2, imaging in 580/60 nm and 670/45 nm optical bands, sampling rate 2.5 Hz).

B. Mild KOH etching to maximize the density of silanol groups on the glass surface (Iler, 1979)

8. Transfer the Piranha-treated coverslips from water to a clean 250 ml polypropylene jar containing 125 ml of 0.5 M KOH, then sonicate for 1 h in a bath sonicator.
   a. Be sure that the water level is up to the marked operating level, and is about level with the solution inside the jar.
   b. Ensure that the temperature does not exceed 40 °C by supplying ice (100~200 g by estimation) at t = 0 and t = 30 min during sonication. Otherwise the bath will heat up, which accelerates etching of the coverslips and creates micron-size pits in the glass surfaces.
   c. Transfer the coverslips back into the polypropylene jar containing water and copiously rinse the coverslips with double-distilled water until pH stabilizes, as verified by pH paper.

C. Silanization to create amine groups on glass surface

Note: In this step, the glass surface is functionalized with amine groups by incubating in a solution of APTES. Although APTES is one the best studied silanization agents, there is currently no consensus on the best method to cost-effectively create molecularly homogenous, reactive, stable APTES films. The parameter space for APTES deposition conditions is vast, as the quality of films has been shown to depend on the substrate (e.g. glass, silica, or oxidized silicon), surface cleaning method, deposition phase (e.g. liquid or vapor), water content of the solvent and the surface, concentration of APTES, temperature, incubation times, and annealing conditions [for references, see Kim et al. (2009)]. Our procedure is based on the simple technique introduced to the single-molecule imaging field by Ha and colleagues (Ha et al., 2002). In our hands, more sophisticated deposition methods did not create APTES surfaces superior to the ones prepared per Ha et al. (2002).

9. Under a fume hood, prepare three clean 125 ml polypropylene jars (maximal capacity of ~170 ml) containing 125 ml acetone.
10. Connect a non-coring needle to a 10 cc gas-tight glass syringe via a PTFE valve. Close the PTFE valve. Insert the needle through the septum of the APTES bottle and ensure that the needle orifice is below the surface of the APTES. Create slight positive pressure inside the APTES sealed bottle using a nitrogen line and a second non-coring needle. Open the PTFE valve. Withdraw 3.75 ml of APTES with the syringe and close

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the PTFE valve. Remove the needle from the bottle, open the PTFE valve, and add the APTES into the 125 ml of acetone in one of the jars. Gently stir to prepare 3% APTES.

11. Using forceps, remove the rack with coverslips from double-distilled water, and dip the rack successively into the two 125 ml polypropylene jars containing acetone, spending ~10 sec in each jar with gentle agitation. Then transfer immediately into the freshly prepared 3% APTES solution. Do not allow acetone to dry between transfers. Place the APTES container on a horizontal platform shaker and incubate for 1 h.

    Note: Immediately after use, during silanization, rinse the syringe, the syringe needles, and the PTFE valve sequentially with acetone and double-distilled water, and then blow with dry nitrogen. Also, during silanization, prepare PEG solutions (steps D14-15).

12. After silanization, wash the coverslips with acetone by dipping the rack in the two jars containing acetone (see step C11) in reverse order and place the rack back into double-distilled water. Copiously rinse with double-distilled water. Do not let the coverslips spend more than 5 min in water. Blow the coverslips dry one-by-one with nitrogen. To that end, hold a coverslip on its corner with the flat-end tweezers, and direct the nitrogen gas flow across the surface of the coverslip towards the corner held by the tweezers. When dry, place the coverslip on a piece of Parafilm positioned on a flat, clean surface. Mark the upper surface of the coverslip by scoring the upper-left corner with a diamond scriber. The coverslips are now ready for PEGylation.

    Notes:

    a. *We usually carry out PEGylation in a clean environment (e.g., a positive-pressure HEPA-filtered room), but we also have had success in a regular environment if PEGylation was carried out immediately after treatment by APTES. We typically do not store amine-treated coverslips.*

    b. *Many APTES deposition protocols include a high-temperature curing step following silanization, meant to form stable covalent bonds between physisorbed APTES molecules* (Plueddemann, 1982)*. However, on the molecular scale, curing may lead to lateral rearrangement of APTES molecules and create islands of unmodified glass (Kim et al., 2009). In our hands, curing offered no additional improvement in surface quality in terms of preventing non-specific adsorption of biomolecules.*

13. Quality control: Concentration of amine groups. You can measure the concentration of amine groups on coverslip surfaces using TRIONE ninhydrin reagent. To that end, crush one coverslip by placing it into a 50 ml Falcon tube and centrifuging at 2,000 *x g* in a swing-bucket centrifuge for 1 min. Then fill the tube with 1 ml of the ninhydrin reagent and follow the manufacturer's protocol to spectrophotometrically quantify the average density of amine groups on the coverslip surface (for 24 x 40 mm coverslips, with the thickness of ~0.15 mm, the total surface area is 1,940 mm^2). Using standard dilutions of APTES as create a calibration curve, we typically get 3 amine groups per nm^2.

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D. Treatment of amine-glass surfaces with NHS-PEG at cloud-point conditions

Notes:

a. The procedure builds upon the method introduced by Ha et al. (2002), with modifications to maximize the density of PEG molecules, and to block and neutralize positively charged amine groups after PEG deposition (section E). Thus, amine-treated coverslips are coupled to succinimidyl-PEG at pH 9.0 in a bicarbonate buffer containing 0.45 M K_2SO_4, at 10.0 % NHS-PEG (w/v). The high salt and the high PEG concentration bring the PEG solution just below its 'cloud point', which maximizes the density of the PEG layer on the surface (Kingshott et al., 2002).

b. NHS group hydrolyzes in aqueous buffers, which makes NHS-PEG non-reactive. The lifetime of the NHS group is between a few seconds and a few minutes, depending on the nature of the NHS-PEG linker, pH, and temperature (Hermanson, 2008). For example, we found that NHS-SVA hydrolyses with a half-life of ~5 min at pH 9.0 at room temperature, which is a good compromise between the reactivity of the NHS group and the reactive species lifetime. Thus, our protocol minimizes the time the reactive NHS-SVA species spends at pH=9.0 prior to addition to the amine-glass surface. Specifically, we initially dissolve dry PEG-SVA at pH 6.0, at which the NHS group remains unhydrolyzed for at least 1 h. Then, immediately before addition of the PEG solution to the glass surface, we bring the pH up to 9.0. If you are using a non-SVA linker, you have to optimize the pH of the reaction to achieve the NHS lifetime of ~5 min.

c. The lifetime of the reactive NHS group can be measured spectrophotometrically by monitoring the kinetics of accumulation of free NHS upon hydrolysis [free NHS strongly adsorbs at 260 nm (Miron, 1982)]. We also highly recommend measuring the percentage of reactive PEG-NHS in new batches of purchased PEG reagents. We have had cases in which completely hydrolyzed, non-reactive PEGs had been shipped by major suppliers.

14. This step is best done during the APTES incubation at step C11. Take out 6 single-use aliquots of dry mPEG-SVA from storage at -80 °C. Each aliquot should be about 5 mg, which is sufficient to treat two coverslips. The mass of dry mPEG-SVA in the 6 aliquots should have been pre-written on each tube with 0.1 mg accuracy prior to storage at -80 °C (*e.g.,* 4.9, 5.0, 5,1, 4.9, 5.0, and 5.1 mg for 6 tubes). In addition, take out 1-2 mg of biotin-PEG-SVA from storage. Let all tubes warm up to room temperature.
15. This step is best done during the APTES incubation at step C11. Calculate the volume of 0.5 M K_2SO_4 solution to add to each mPEG-SVA tube by multiplying the aliquot mass by 8 (*e.g.,* the tube containing 4.9 mg will require 4.9 x 8 = 39.2 µl of K_2SO_4 solution) and record all volumes in a notebook. Sum up all the volumes, to get the minimal volume of 0.5 M K_2SO_4 required to make six PEG solutions [*e.g.*, for 6 tubes containing 4.9, 5.0, 5,1, 4.9, 5.0, and 5.1 mg mPEG-SVA one needs (4.9 + 5.0 + 5.1 + 4.9 + 5.0 + 5.1) x 8 = 240 µl of K_2SO_4]. Based on the calculated minimal volume, prepare sufficient amount of 0.5%

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biotin-PEG-SVA solution in 0.5 M K_2SO_4 (*e.g.,* dissolve 1.3 mg of biotin-PEG-SVA with 260 µl 0.5 M K_2SO_4). Pipet the pre-calculated volumes of the 0.5% biotin-PEG-SVA solution into each of the 6 mPEG-SVA aliquots (*e. g.* add 39.2 µl to the 4.9 mg tube, and so on), vortex for 10 sec, and briefly spin with a tabletop centrifuge. Each tube now should have a clear solution and a small (~5 µl in volume) pellet of PEG on the bottom.

Note: PEG precipitation is expected, because the PEG solution is currently at 11.6% concentration (w/v), which is above its 'cloud point' (10% w/v).

The tubes can remain at room temperature for at least 1 h without appreciable hydrolysis of the NHS group while the coverslips are being treated with APTES and laid out for PEGylation.

16. With all APTES-treated coverslips laid out for PEGylation, set up the PEGylation reactions using one PEG-NHS aliquot at a time. To that end, add 1/8 of 1 M $NaHCO_3$ (pH 9.0) to the PEG solution in K_2SO_4 (*e.g.* add 4.9 µl of $NaHCO_3$ to a tube that originally contained a 4.9 mg mPEG-SVA aliquot, and now contains 39.2 µl of the biotin-PEG-SVA solution), quickly mix by pipetting up and down, and deposit the whole solution in the center of a dry APTES-treated coverslip. Cover the drop with another coverslip carefully, making sure that the scored surfaces of both coverslips face towards the PEG solution, and avoiding formation of bubbles (by practicing). Repeat for the rest five pairs of coverslip and incubate for 30 min at room temperature.

Note: The PEG solution appears clear after addition of $NaHCO_3$, because the total PEG concentration is now just below the 'cloud point'. We noticed that additional coating of coverslips with a fresh solution of PEG does not improve the quality of surfaces (in terms of non-specific adsorption of biomolecules).

17. Carefully separate the coverslip pairs using tweezers and place individual coverslips back into the ceramic rack in the double-distilled water. Rinse extensively with water until there is no more foaming of the solution.

    Quality control: Density of PEG molecules. You can estimate the packing density of the PEG molecules on the glass surface by depositing fluorescein-PEG-NHS in the same conditions as described above, and measuring the absorbance at 494 nm using a high-sensitivity double-beam spectrophotometer (*e.g.,* Perkin Elmer Lambda 35). The molecules density = [(OD_{494}/extinction coefficient) x 6.02 x 10^{23} mol^{-1}]/area. By this estimation, the average packing density should be consistent with the radius of gyration of a M_w = 5,000 PEG molecule at cloud point [R = 2.8 nm (Dalsin *et al.*, 2005)].

E. Blocking of unreacted amines with acetyl groups

Note: Due to the bulkiness of PEG molecules, only a fraction of amine groups on the surface reacts with NHS-PEG. As a result, the remaining amines contribute to non-specific adsorption of negatively charged molecules, such as DNA, which is problematic in buffers of low ionic strengths. Thus, we cap the unreacted amines with NHS-acetate to convert them to neutral, stable amides.

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18. Blow-dry 12 PEGylated coverslips as described in step C12. Place dry coverslips on Parafilm, with the PEGylated surface facing up.
19. Immediately prior to use, dissolve 3 mg of sulfo-NHS-acetate in 300 µl of 0.1 M $NaHCO_3$ (pH 9.0), deposit 50 µl drops of the solution onto the 6 PEG coverslips, and cover with the remaining 6 coverslips, with the PEGylated surface facing the solution. Incubate for 30 min at room temperature. Rinse coverslips with double-distilled water, blow-dry, and store dry at -80 °C.

F. Testing fluorescent background of PEGylated surfaces and non-specific absorption of biomolecules

Note: The following protocol is for a rapid test of non-specific 'stickiness' of the surface to fluorescently labeled biomolecules. However, this test does not address the biological activity of surface-immobilized molecules (for instance, the activity of a transcription factor on a surface-immobilized DNA). Thus, the most relevant test of surface quality is a biochemical assay of the activity of surface-bound biomolecule, performed side-by-side with a positive control in which the activity of the same amount of biomolecules is measured in solution-based conditions (Revyakin et al., 2012).

20. Create 5 open wells on a coverslip using 1 mm-wide strips of double-sided tape (by simply sticking 6 strips on to the modified side), and place the coverslip on an objective-type TIRF microscope. The 5 wells allow testing of 5 different biomolecules.
21. Deposit a 5 µl drop of PBST into a well, turn on the excitation laser, and focus the microscope onto the surface. Acquire a movie to measure the fluorescent background on the surface, under single-molecule imaging conditions (*e.g.,* excitation with 532 and 640 nm sources at density ~0.5 kW/cm^2, imaging in 580/60 nm and 670/45 nm optical bands, sampling rate 2.5 Hz). In our hands, silanization and PEGylation increase the number of fluorescent background spots ~3 fold in comparison to Piranha-treated glass (*i.e.,* an increase from ~3 spots to ~10 spots per 100 x 100 µm field of view), which is an acceptable level of background for most single-molecule imaging experiments. Allow the background spots to photobleach.
22. Prepare a 10 nM solution of the fluorescently labeled test protein in PBST, and deposit 20 µl of the solution on top of the 5 µl PBST drop already in the well. Acquire a movie to quantify the non-specific adsorption of the molecule to the surface. For simplicity, in this test we do not supply oxygen scavenger to the solution of test protein. Thus, to avoid immediate photobleaching upon sticking of a molecule to the surface (which will lead to over-estimation of surface quality) use minimal laser power (30~100 W/cm^2) and fluorescent labels that photobleach in >5 seconds in the absence of oxygen scavengers (*e.g.,* Cy3 and Atto633). For a good-quality surface, we typically observe a 'cloud' of 10 nM biomolecules rapidly diffusing in the bulk solution (at 2.5 Hz acquisition rate), and ~10 single-molecule limited spots in any given movie frame (100 x 100 µm field of view). No additional accumulation of spots should be observed within 10 min

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(with the excitation light switched off, and then turned back on again), indicating that non-specific adsorption is rare and reversible. Repeat the test with other biomolecules of interest using the remaining 4 wells.

Recipes

1. 0.5 M KOH (per liter)
   28 g KOH
   Note: Solution can be re-used.
2. 0.5 M K_2SO_4 (pH 6.0) (per liter)
   174 g K_2SO_4
   Note: Store at room temperature. If your double-distilled water supply is acidic, no pH adjustment of the K_2SO_4 solution is necessary. If your double-distilled water supply is basic, adjust pH to 6.0 using diluted H_2SO_4.
3. 1 M $NaHCO_3$ (pH 9.0) (10x) (per liter)
   82 g $NaHCO_3$
   Note: Adjust pH to 9.0 with NaOH and store in single-use aliquots at -80 °C.
4. Phosphate-buffered saline containing 0.1% Tween (PBST) (per liter)
   2.0 g KCl
   2.4 g KH_2PO_4
   80 g NaCl
   14.4 g Na_2HPO_4
   1 ml Tween 20

Acknowledgments

We thank the groups of Steven Chu and Robert Tjian for support (University of California, Berkeley, and Janelia Farm Research Camus, supported by the NIH grant 1P01CA112181-01A1 and Howard Hughes Medical Institute).

References

1. Axelrod, D. (1981). Cell-substrate contacts illuminated by total internal reflection fluorescence. *J Cell Biol* 89(1): 141-145.
2. Dalsin, J. L., Lin, L., Tosatti, S., Voros, J., Textor, M. and Messersmith, P. B. (2005). Protein resistance of titanium oxide surfaces modified by biologically inspired mPEG-DOPA. *Langmuir* 21(2): 640-646.
3. Ha, T., Rasnik, I., Cheng, W., Babcock, H. P., Gauss, G. H., Lohman, T. M. and Chu, S. (2002). Initiation and re-initiation of DNA unwinding by the Escherichia coli Rep helicase. *Nature* 419(6907): 638-641.

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4. Hermanson, G. T. (2008). Bioconjugation techniques. *Academic Press*, 10, p.0123705010.
5. Iler, R. K. (1979). The chemistry of silica: solubility, polymerization, colloid and surface properties, and biochemistry. *Wiley* 896.
6. Kim, J., Seidler, P., Wan, L. S. and Fill, C. (2009). Formation, structure, and reactivity of amino-terminated organic films on silicon substrates. *J Colloid Interface Sci* 329(1): 114-119.
7. Kingshott, P., Thissen, H. and Griesser, H. J. (2002). Effects of cloud-point grafting, chain length, and density of PEG layers on competitive adsorption of ocular proteins. *Biomaterials* 23(9): 2043-2056.
8. Miron, T. and Wilchek, M. (1982). A spectrophotometric assay for soluble and immobilized N-hydroxysuccinimide esters. *Anal Biochem* 126(2): 433-435.
9. Ostuni, E., Chapman, R. G., Holmlin, R. E., Takayama, S. and Whitesides, G. M. (2001). A survey of structure - property relationships of surfaces that resist the adsorption of protein. *ASC Publications* 17(9): pp.5605-5620.
10. Plueddemann, E. (1982). *Silane coupling agents*, Springer Netherlands.
11. Prime, K. L. and Whitesides, G. M. (1993). Adsorption of proteins onto surfaces containing end-attached oligo(ethylene oxide): a model system using self-assembled monolayers. *J Am Chem Soc* 115(23): 10714-10721.
12. Revyakin, A., Zhang, Z., Coleman, R. A., Li, Y., Inouye, C., Lucas, J. K., Park, S. R., Chu, S. and Tjian, R. (2012). Transcription initiation by human RNA polymerase II visualized at single-molecule resolution. *Genes Dev* 26(15): 1691-1702.
13. Selvin, P. R. and Ha, T. (2008). Single-molecule techniques : a laboratory manual. Cold Spring Harbor Laboratory Press.
14. Visnapuu, M. L., Duzdevich, D. and Greene, E. C. (2008). The importance of surfaces in single-molecule bioscience. *Mol Biosyst* 4(5): 394-403.
15. Weiss, S. (1999). Fluorescence spectroscopy of single biomolecules. *Science* 283(5408): 1676-1683.
16. Zhang, Z., Revyakin, A., Grimm, J. B., Lavis, L. D. and Tjian, R. (2014). Single-molecule tracking of the transcription cycle by sub-second RNA detection. *Elife* 3: e01775.