Functionalization of AFM tips with Biotin

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AFM tips with Biotin

short version

for risks and details see full length procedure

Preparation of tips with biotin linker

1. Aminofunctionalization of the cantilever(s) (see *AFM_tip_aminofunctionalization*).
2. Dissolve 1 portion of Biotin-PEG-NHS (1 mg) in chloroform (0.5 mL), transfer the solution into the reaction chamber, add triethylamine (30 µL) and mix.
3. Immediately place cantilever(s) in the reaction chamber, cover the chamber and incubate for 2 h.
4. Wash with chloroform (3 × 10 min), dry with nitrogen gas. Store for several weeks under argon or continue with the next step.

Dense coverage of mica with avidin (for force spectroscopy)

1. Cleave mica sheet.
2. Mount sheet in fluid cell
3. Add 200 µL avidin working solution **1** (0.1 mg/mL in ~15 mM NaCl), incubate for 15 min.
4. Rinse with 1 mM NaCl (10 ×).
5. Rinse with PBS (10 to 50 ×).
6. Keep wet, proceed to AFM measurements in PBS.
7. Use free biotin (0.1 mg/mL = 0.4 mM) or free streptavidin (0.05 mg/mL) for specific block of the avidin-biotin interactions.

Sparse coverage of mica with avidin (for TREC microscopy)

1. Cleave mica sheet.
2. Mount sheet in fluid cell
3. Add 200 µL avidin working solution **2** (2.5 µg/mL in ~1 mM NaCl), incubate for 20 min.
4. Rinse with 1 mM NaCl (10 ×).
5. Rinse with PBS (10 to 50 ×).
6. Keep wet; proceed to AFM measurements in PBS.
7. Use free streptavidin (0.05 mg/mL) for specific block of the avidin-biotin interactions.

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Functionalization of AFM tips with Biotin

Please, read the manual *general_overview* for the basic concept of AFM tip functionalization with long flexible PEG linkers.

Please, read the manual *AFM_tip_aminofunctionalization* for the details on how amino groups are generated on the AFM tip surface.

Novices in recognition force spectroscopy/microscopy need a simple test system which is easy to prepare and yields clear-cut data. For this purpose, biotin is tethered to the AFM tip via a polyethylene glycol (PEG) chain that has a length of 6-9 nm (PEG18 or PEG27). Biotin (also termed vitamin H) is a very specific ligand of avidin, a minor protein component of egg white. In fact, the affinity between biotin and avidin is the highest known in the whole biosphere ($K_D \sim 10^{-15}$ M, dissociation half time ~ 200 days [Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol. 184*, 51-67]). When free biotin is linked to a PEG chain, the affinity is reduced. Nevertheless, the dissociation half time is still on the order of days [Gruber et al., 1997; Kaiser et al., 1997; Marek et al., 1997].

The high affinity between avidin and biotin is not the only favorable aspect. The second advantage is that PEG linkers are available which already contain **biotin** on one end and the desired NHS ester function on the other. Our "Biotin-PEG-NHS" molecules have PEG chains with 18 or 27 ethylene glycol units (see **Figure 1**) because these chain lengths proved optimal in recognition force spectroscopy and microscopy [Riener et al., 2003; Ebner et al., 2005]. Other versions of Biotin-PEG-NHS are commercially available but usually with much shorter or longer PEG chains.

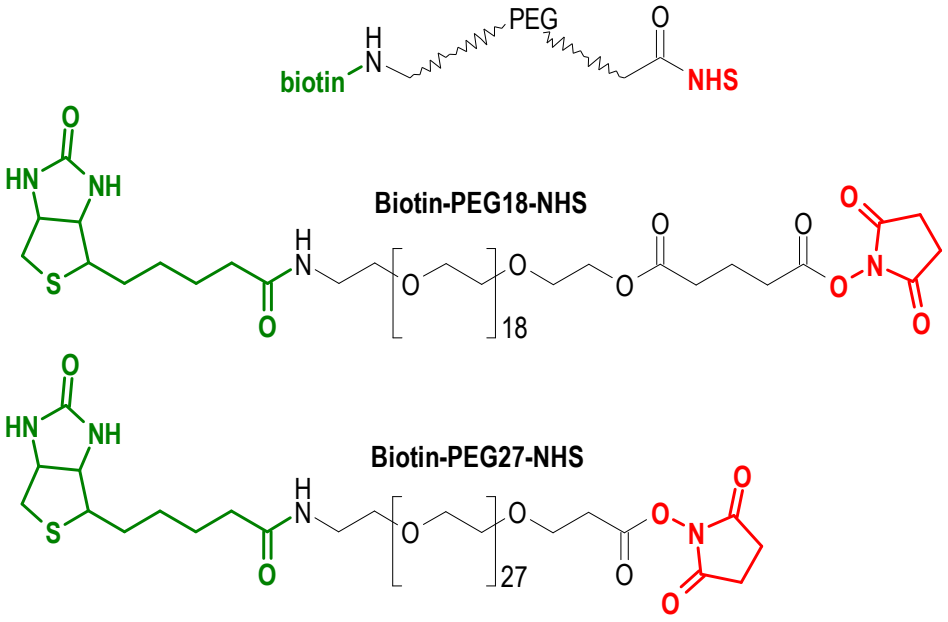

Figure 1: Structure of the Biotin-PEG18-NHS and Biotin-PEG27-NHS which are used to tether biotin residues to amino-functionalized AFM tips (see **Figure 2**).

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As depicted in **Figure 2**, the first step of tip functionalization is the generation of amino groups on the tip surface and the second step is the reaction of the amino groups with the NHS ester function of the PEG linker (Biotin-PEG-NHS).

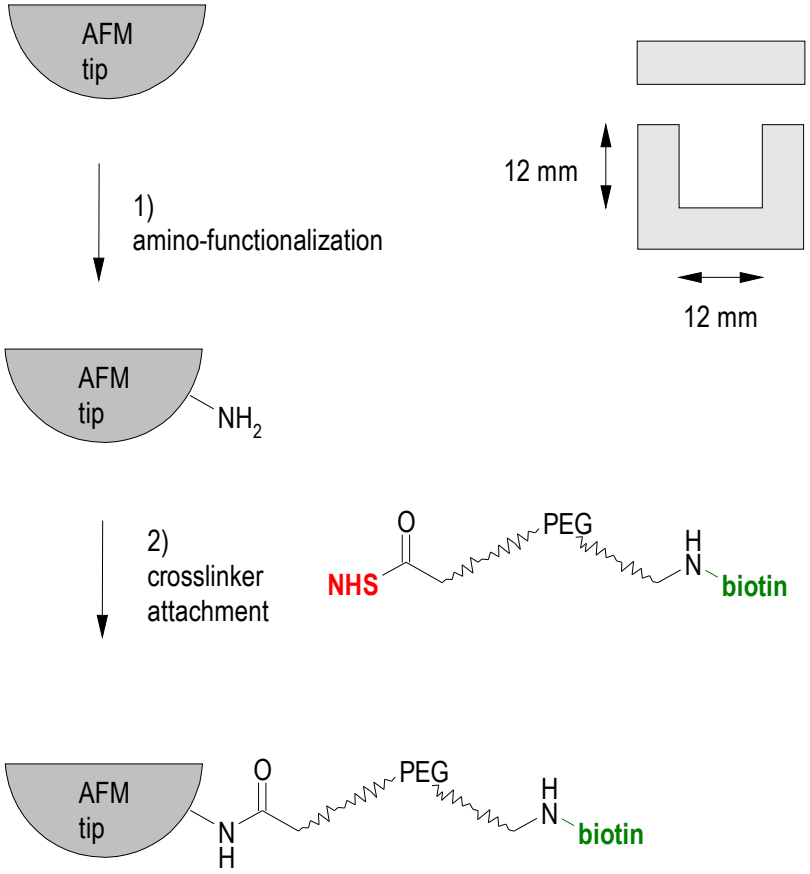


Figure 2: Reaction of amino-functionalized AFM tips with Biotin-PEG-NHS results in flexibly bound biotin which has high affinity for avidin or streptavidin.

The experimental details for the **first step** are described in the manual *AFM_tip_aminofunctionalization*.

For the second step, Biotin-PEG-NHS (1 mg, i.e., the contents of one crimp-sealed vial) is dissolved in chloroform (0.5 mL) and the solution is transferred into a tiny reaction chamber. Such a chamber can be prepared by drilling a circular hole with a perfectly flat bottom into a small Teflon block (see sketch in **Figure 2**). The chamber is covered with a suitable Teflon disk. Alternatively, a graduated 10 mL measuring cylinder can be cut between the 1 and 2 mL marks and the rim is smoothened with a hot flame. Triethylamine (30 µL) is added and mixed into the solution. The amino-functionalized tips are immersed in this solution and the reaction chamber is covered (with a suitable Teflon disk, or an inverted small glass beaker, or with aluminum foil) to minimize evaporation of chloroform and triethylamine. After two hours the tips are washed in chloroform (3 × 10 min incubation) and dried with nitrogen gas. The biotin-functionalized tips can be stored in an argon-filled desiccator for several weeks.

Force spectroscopy experiments

are performed as described in Riener et al. (2013, see reference 1 on the last page). An important control experiment is the specific block of the avidin-biotin interaction, either with free biotin (0.1 mg/mL = 0.4 mM, [Riener et al., 2003]) or with free streptavidin (0.05 mg/mL = 0.83 µM, [Ebner et al., 2005]) in the measuring buffer.

Free biotin will block the biotin-binding sites of all mica-bound avidin molecules. For this purpose, the proper volume of a biotin stock solution (10 mM, see Notes, below) is added to the measuring buffer to reach a final biotin concentration of 0.4 mM. For instance, 20 µL of the 10 mM biotin stock solution is added to 500 µL bath volume.

Free streptavidin will block the biotin groups on the AFM tip. For this purpose, the proper volume of a streptavidin stock solution (1 mg/mL, see Notes, below) is added to the measuring buffer to reach a final biotin concentration of 0.05 mg/mL. For instance, 25 µL of streptavidin stock solution (1 mg/mL) is added to 500 µL bath volume.

Recognition force microscopy experiments

are performed as described in Ebner et al. [2005]. The specific block is performed as described in the force spectroscopy experiments, usually with free streptavidin [Ebner et al., 2005].

Notes:

- Pipetting of chloroform and triethylamine should be performed with glass pipettes and a pipette bulb (or with a graduated glass capillary in case of triethylamine) and not with the plastic tips of digital pipettes because the plastic tips may release polymeric material in chloroform and triethylamine. – Never use your mouth for pipetting, especially not with such toxic liquids as chloroform and triethylamine.
- The ideal tools for accurate and easy pipetting of chloroform and triethylamine are gas-tight Hamilton syringes. A 500 µL syringe is suggested for chloroform (part no. 81230, RN-type) and a 50 µL syringe for triethylamine (part no. 80230, RN-type). Two removable needles with a blunt ends (part no. 7780-02) need to be purchased in addition.
- Immediately after pipetting, these syringes should be washed free of chloroform or triethylamine. Use nitrile or rubber gloves (not latex) for the cleaning procedure. First, isopropanol (same as 2-propanol) is pulled into the syringe and disposed into the solvent waste (3 ×). Then, the piston is removed and carefully rinsed with isopropanol. The barrel is filled with isopropanol several times from a squeeze bottle and the contents poured into the solvent waste. Finally, the barrel is again filled with isopropanol and the piston is inserted to push the contents into the solvent waste bottle. This step is repeated twice. Subsequently, the syringe must be dried by one of two alternative methods:
    - Diethyl ether can be pulled into the syringe and pushed into the solvent waste. Then the piston is moved up and down (15-20 ×).

- The outsides of the barrel and the piston are dried with soft paper tissue or with a stream of gas. The barrel interior is dried with nitrogen gas from a Pasteur pipette which is inserted into the barrel. Then, the needle is inserted into the tip of the Pasteur pipette until the glass base of the syringe needle softly touches the tip of the Pasteur pipette. Now the gas flow is forced through the needle which causes evaporation of isopropanol from the interior of the needle (takes about 1 min).

- Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing. In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.
- In spite of the small quantities of chloroform and triethylamine, the reactions should be performed in a well ventilated hood.
- If your institution forbids the use of chloroform, then you can use DMSO for the reaction of the tip with the linker and isopropanol (or ethanol) for the subsequent washing steps. Test experiments have shown that this gives good results.
- Unfortunately we have no experience whether chloroform can be replaced by some other reagent in the initial washings, before aminosilanization. Chlorinated solvents are much more efficient in the removal of impurities. You can probably use dichloromethane if that is allowed at your institution. If not, then it may be better to use ozone cleaning or piranha, followed by washing with water and then with isopropanol or ethanol.
- Strictly avoid contact of the organic solvents and of triethylamine with your skin. Latex gloves cannot protect you for more than 1-2 seconds. Nitrile gloves provide slightly longer protection. In case of splashing the gloves must be immediately removed from the hand.
- The 10 mM biotin stock solution is sensitive to oxidation by air on a time scale of few hours. It must quickly be divided into many small aliquots and stored frozen at -25°C. The aliquots should be kept frozen until few minutes before their use in the experiments. You are allowed to thaw a large aliquot, divide it into many small aliquots and quickly freeze it again. In contrast to protein samples, it is not the number of freezing/thawing cycles but the time at room temperature which determines the extent of degradation. You may ask why free biotin is so sensitive to oxidation by air while tip-bound biotin is not. One possible answer is that the free COOH group of biotin can bind traces of transition metal ions (e.g. $Fe^{2+/3+}$ from the glassware surface) which may act as catalysts for spontaneous oxidation of the sulfur atom in biotin.

After functionalization of the cantilever with biotin, a complementary receptor protein (avidin or streptavidin) must be permanently fixed onto an ultra-flat sample surface. The easiest and fastest way is electrostatic adsorption of avidin to freshly cleaved mica [Riener et al., 2003]. The mica surface is negatively charged and avidin is among the few proteins which have a

large excess of positive surface charges at neutral pH (other examples of cationic proteins are lysozyme or histones). The adsorption is particularly fast and strong at low ionic strength, therefore the adsorption of avidin on mica is performed at low NaCl concentrations (not in physiological buffer which contains 150 mM NaCl). Once adsorbed, the avidin molecules remain bound to mica even in physiological buffer (such as PBS).

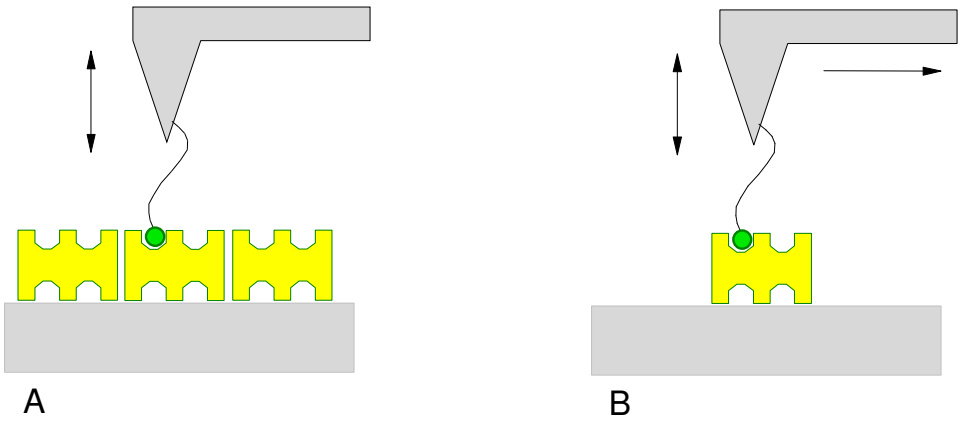


Figure 3: Model experiments with biotin-functionalized tips. (A) Force spectroscopy requires a high lateral density of avidin on mica in order to maximize the probability for avidin-biotin recognition in each force-distance cycle. (B) Simultaneous Topography and RECognition (TREC) microscopy requires a low density of avidin on mica in order to allow for lateral resolution of individual avidin molecules.

Phosphate-buffered saline (PBS) is prepared by dissolving the components listed below in about 800 mL water, transferring the solution into a 1 L volumetric flask and adding water to give exactly 1 L volume. Mix carefully. The pH will automatically be 7.3 if all steps are performed correctly. No pH adjustment is required. Aliquots can be stored at -20°C for up to several years.

- 140 mmol NaCl (58,44 g/mol × 0,140 mol = **8.182 g NaCl**)
- 2.7 mmol KCl (74.56 g/mol × 0.0027 mol = **0.201 g KCl**)
- 10 mmol Na_2HPO_4 (in case of anhydrous dibasic sodium phosphate: 141.96 g/mol × 0.010 mol = **1.420 g anhydrous Na_2HPO_4**; in case of the heptahydrate: 268.07 g/mol × 0.010 mol = **2.681 g $Na_2HPO_4.7H_2O$**)
- 1.8 mmol KH_2PO_4 (136.09 g/mol × 0.0018 mol = **0.245 g anhydrous KH_2PO_4**).

100 mM NaCl is prepared by dissolving 10 mmol NaCl (**584 mg**) in water at a final volume of 100 mL. Aliquots of 1 mL are frozen in 1.5 mL reaction vials and stored at -20°C.

1 mM NaCl is prepared by thawing a 1 mL aliquot of 100 mM NaCl (see previous paragraph) and diluting it with 99 mL water.

Avidin stock solution (1 mg/mL): Avidin (~2 mg) is weighed into a screw-cap vial (4 mL or 8 mL) and PBS is added to give a final avidin concentration of exactly 1 mg/mL.

Aliquots of 20 µL are carefully pipetted into the bottom of 0.5 mL reaction vials, shock-frozen in liquid nitrogen, and stored at -20°C or -80°C for up to several years.

Avidin working solution **1** (0.1 mg/mL): One portion of the frozen avidin stock solution (20 µL 1 mg/mL) is taken out of the freezer and mixed with 180 µL of 1 mM NaCl which causes thawing. This solution must be used within one workday.

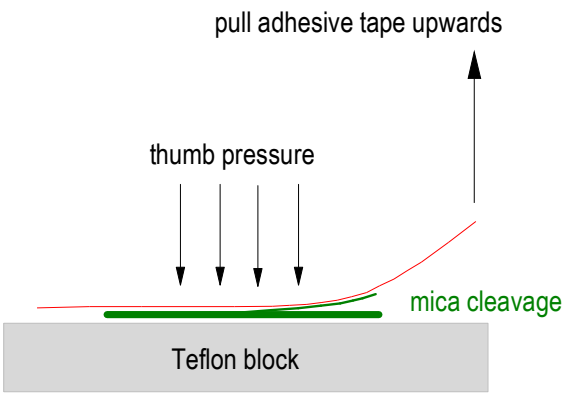
Avidin working solution **2** (2.5 µg/mL): One portion of the frozen avidin stock solution (20 µL 1 mg/mL) is taken out of the freezer, thawed, and well mixed with a pipette tip. One 2.5 µL aliquot of this solution is transferred into 1 mL 1 mM NaCl and the resulting solution is mixed with a 1 mL pipette tip. This dilute solution must be used immediately.

50 mM Na_2HPO_4 is prepared by dissolving the proper amount of Na_2HPO_4 in water. The amount of Na_2HPO_4 depends on whether you have purchased the heptahydrate ($Na_2HPO_4.7H_2O$, 268.3 g/mol) or the dihydrate ($Na_2HPO_4.7H_2O$, 177.99 g/mol). Dissolve 1.555 g of the heptahydrate or 0.890 g of dihydrate in water at a final volume of 100 mL to achieve a phosphate concentration of 50 mM. Do not adjust the pH! If you check it you will find pH > 8. This is important for dissolving biotin which is acidic (see next paragraph).

10 mM biotin stock solution (2.443 mg/mL): Weigh less than 120 mg of biotin into a 50 ml measuring cylinder. Divide the actual mass by the desired concentration (2.443 mg/mL) to obtain the desired volume. Add 50 mM Na_2HPO_4 (pH > 8, see previous paragraph) to reach this desired volume. Cover with Parafilm and mix well. The final pH will be between 7.0 and 7.5, which is fine. At this stage, biotin is sensitive to oxidation by air on a time scale of few hours. You should immediately divide the 10 mM stock solution of biotin into small aliquots (e.g. 200 µL) and store them frozen at -25°C.

1 mg/mL streptavidin stock solution: Streptavidin is usually purchased in a quantity of 1 mg. This amount is dissolved in 1 mL of buffer and divided into 50 µL aliquots which are initially frozen in liquid nitrogen and then stored at -25°C. You can use PBS as buffer. Even better would be the basic buffer recommended by commercial providers because it assures to keep streptavidin in a monomeric state during freezing and thawing.

Cleavage of mica: The procedure is performed with clean, powder-free latex or nitrile gloves. A sheet of mica is cut with scissors to fit into the fluid cell. The sheet is placed on a clean Teflon block with the "better" side facing downwards. A strip of adhesive tape (e.g., Scotch Tape) is pressed onto the mica sheet so that it sticks to mica and has overhanging ends (see sketch below). Then, the mica sheet is pressed onto the Teflon block with the thumb while pulling the longer end of the adhesive tape upwards. The adhesion between mica and adhesive tape is stronger than cohesion inside the mica sheet, causing cleavage of the mica sheet. The adhesive tape with the mica layer is discarded. Subsequently, the mica sheet is turned upside down and the cleavage process is repeated on the second side



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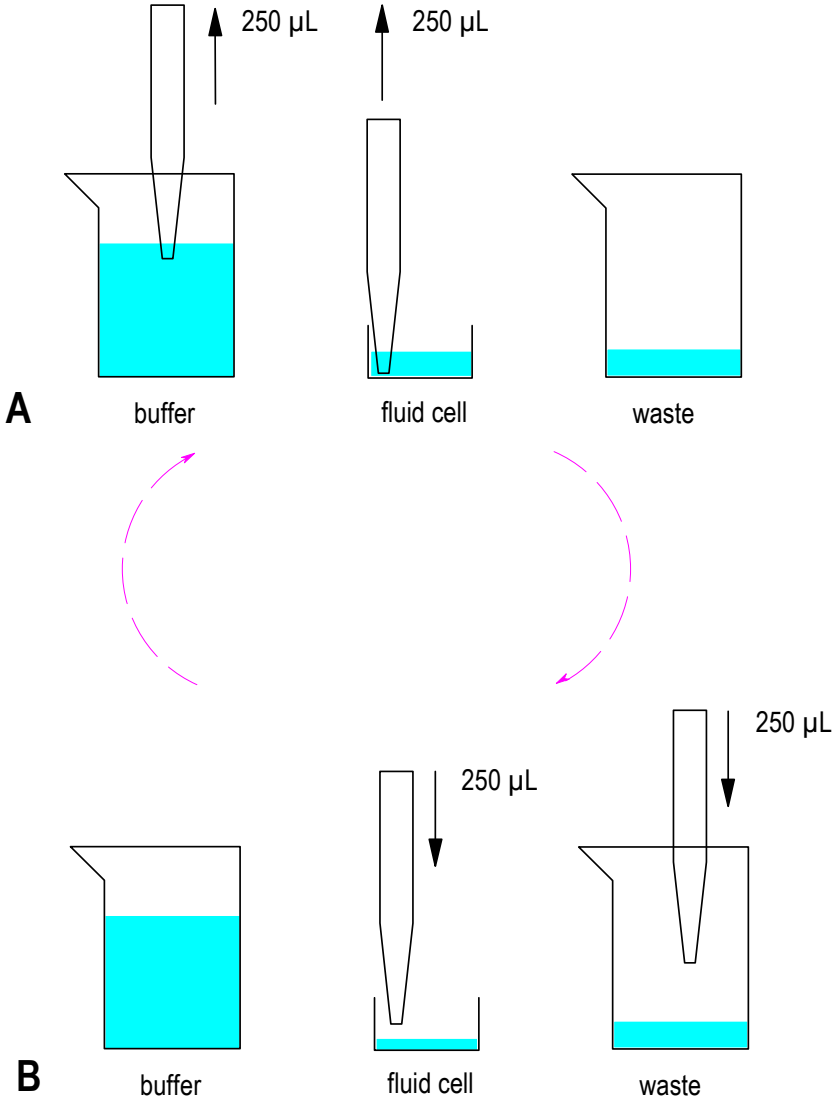
of the mica sheet. The Teflon block is washed with ethanol and stored in a dust-free box.

Adsorption of avidin to mica (one of two alternative methods):

1. Adsorption of avidin at high density (for force spectroscopy): The freshly cleaved mica sheet is immediately mounted in the fluid cell and 200 µL of the avidin working solution **1** is pipetted into the cell and allowed to sit for 15 min. Then, the surface is rinsed with 1 mM NaCl (10 ×) and with PBS (between 10 × and 50 ×).

   The rinsing cycles with 1 mM NaCl and with PBS are performed as follows. First, 150 µL of 1 mM NaCl is added into the fluid cell, resulting in a final volume of 350 µL. Then, you take a digital pipette into each hand, both pipettes being adjusted to a volume of 250 µL (see sketch below). In step A, the left pipette is loaded with fresh buffer (250 µL) and at the same time the right pipette withdraws 250 µL of buffer from the fluid cell. In step B, the left pipette delivers its content into the fluid cell and the right pipette into the waste beaker.



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The surface must be kept wet during mounting in the AFM and during all other times. Measurements are performed in PBS.

2. Adsorption of avidin at low density (for TREC microscopy): A droplet of the avidin working solution **2** is placed onto the freshly cleaved mica surface and allowed to sit for 20 min. Then, the surface is rinsed with 1 mM NaCl (10 ×) and with PBS (between 10 × and 50 ×, see previous paragraph and the sketches below). The surface must be kept wet during mounting in the AFM. Measurements are performed in PBS.

Literature on model experiments with biotin-functionalized AFM tips:

Riener, C. K., Stroh, C. M., Ebner, A., Klampfl, C., Gall, A. A., Romanin, C., Lyubchenko, Y. L., Hinterdorfer, P., and Gruber, H. J. (2003) Simple test system for single molecule recognition force microscopy. Anal. Chim. Acta 479, 59-75.

Ebner, A., Kienberger, F., Kada, G., Stroh, C. M., Geretschläger, M., Kamruzzahan, A. S. M., Wildling, L., Johnson, W. T., Ashcroft, B., Nelson, J., Lindsay, S. M., Gruber, H. J., and Hinterdorfer, P. (2005) Localization of single avidin-biotin interactions using simultaneous topography and molecular recognition imaging. *ChemPhysChem. 6*, 897-900.

Green, N. M. (1990) Avidin and Streptavidin. *Methods Enzymol. 184*, 51-67.

Gruber, H. J., Marek, M., Schindler, H., and Kaiser, K. (1997) Biotin-fluorophore conjugates with poly(ethylene glycol) spacers retain intense fluorescence after binding to avidin and streptavidin. *Bioconjugate Chem. 8,* 552-559.

Kaiser, K., Marek, M., Haselgrübler, T., Schindler, H., and Gruber, H. J. (1997) Basic studies on heterobifunctional biotin-PEG conjugates with a 3-(4-pyridyldithio)propionyl group on the second terminus. *Bioconjugate Chem. 8,* 545-551.

Marek, M., Kaiser, K., and Gruber, H. J. (1997) Biotin-pyrene conjugates with poly(ethylene glycol) spacers are convenient fluorescent probes for avidin and streptavidin. *Bioconjugate Chem. 8,* 560-566.

Risk and Safety

	**Biotin-PEG-NHS,** chemically homologous to old-style Biotin-PEG-NHS, CAS No. 565471-88-7, unknown hazard potential. Warning: all biotin derivatives should be handled with great precaution. For example, iminobiotin is known to be highly neurotoxic!
	**Chloroform** ($CHCl_3$): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37

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	**Diethyl ether,** extremely flammable, narcotic, forms explosive peroxides when allowed to evaporate, H224-H302-H336, P210-P261, R12-19-22-66-67 (Europe), S9-16-29-33 (Europe)
	**Isopropanol = 2-propanol:** flammable, H225-H319-H336, P210-P261-P305 + P351 + P338, R11-36-67 (Europe), S7-16-24/25-26 (Europe)
	**Triethylamine** (TEA): flammable, irritant, caustic, H225-H302-H312-H314-H332, P210-P280-P305 + P351 + P338-P310, R20/21/22-35, S3-6-26-29-36/37/39-45

Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing. In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.

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