

# Functionalization of AFM tips with Aldehyde Linkers

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5. Scope: The procedures have been optimized for AFM tip functionalization. They may work in related fields but the optimal parameters may be different. For instance, much slower coupling will occur on protein-resistant surfaces.
6. You are kindly asked for feed-back concerning errors, unexpected results, or potential hazards not foreseen at present.

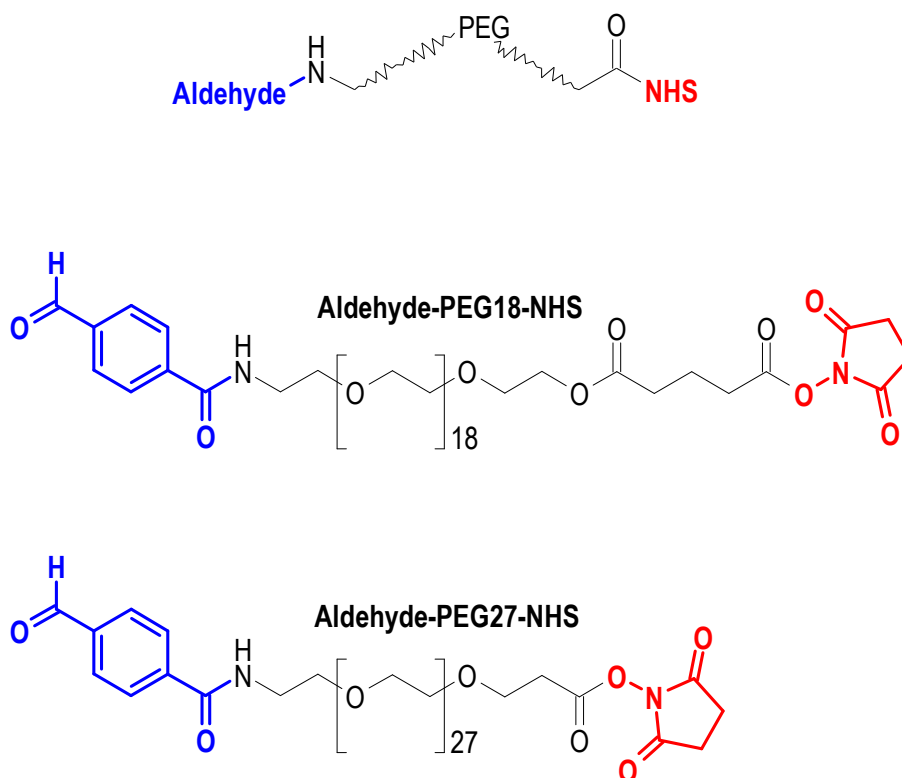
**AFM tips with Aldehyde**  
**short version**  
**for risks and details see full length procedure**

1. Aminofunctionalization of the cantilever(s) (see [AFM\\_tip\\_aminofunctionalization](#)).
2. Dissolve 1 portion of Aldehyde-PEG-NHS (3.3 mg) in chloroform (0.5 mL), transfer the solution into the reaction chamber, add triethylamine (30  $\mu$ L) and mix.
3. Immediately place cantilever(s) in the reaction chamber, cover the chamber and incubate for 2 h.
4. Wash with chloroform (3  $\times$  10 min), dry with nitrogen gas. Continue with the next step.
5. Freshly prepare a 1 M solution of sodium cyanoborohydride (with 20 mM NaOH) from the following components.
  - 13 mg NaCNBH<sub>3</sub> (or 32 mg) – **toxic!**
  - 20  $\mu$ L 100 mM NaOH (or 50  $\mu$ L)
  - 180  $\mu$ L water (or 450  $\mu$ L)
6. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
7. Pipet 100  $\mu$ L protein solution (1-2  $\mu$ M) onto the cantilever(s).
8. Add 2  $\mu$ L of the 1 M sodium cyanoborohydride stock solution, mix carefully, cover with lid, incubate for 1 h.
9. Meanwhile dissolve 3 pellets NaOH in 500 mL water, add the unused NaCNBH<sub>3</sub> solution, mix, pour into the drain and flush with tap water.
10. Add 5  $\mu$ L of ethanolamine (1 M, pH 8.0) to the drop on the cantilever(s), mix cautiously, cover with lid, incubate for 10 min.
11. Wash in PBS or any other buffer of choice (3  $\times$  5 min)
12. Mount cantilever in AFM setup (or store in a 24 well plate under PBS at 4°C for 1-2 weeks)

## Functionalization of AFM tips with Aldehyde Linkers

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.

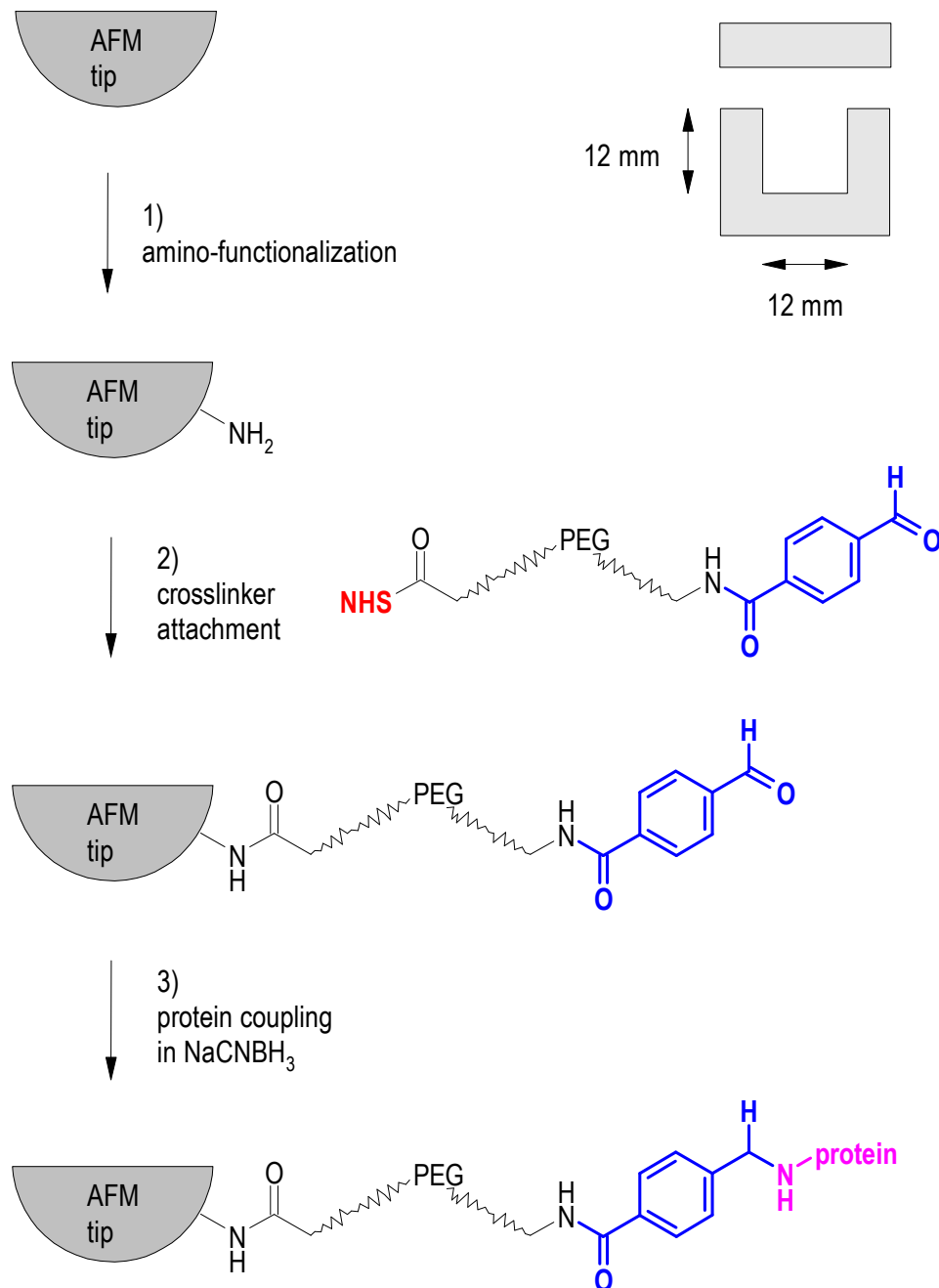


**Figure 1:** Structure of the Aldehyde-PEG18-NHS and Aldehyde-PEG27-NHS which are used to tether proteins to amino-functionalized AFM tips (see **Figure 2**).

As depicted in **Figure 2**, the first step of tip functionalization is the generation of amino groups on the tip surface, the second step is the reaction of the amino groups with the NHS ester function of the PEG linker (Aldehyde-PEG-NHS), and the third step is coupling of the protein [Ebner et al., 2007].

Besides proteins, other molecules with amino groups can also be coupled to the aldehyde functions on the AFM tip. Hereby it is important to consider the peculiar mechanism of protein coupling to AFM tips. Protein coupling to AFM tips is accelerated by at least 1000-fold, due to slight pre-adsorption of protein to the tip (see manual "[acceleration\\_of\\_coupling\\_by\\_preadSORPTION](#)"). This acceleration may be absent when coupling other molecules with amino groups. For this reason we used 5 mM of amine-modified ATP derivative in tip derivatization [Wildling et al., 2011] (in contrast to 0.15 mg/mL = 1  $\mu$ M antibody).

We have never tried coupling of amine-modified oligonucleotides to aldehyde tips. Given the choice we would rather prefer to couple thiol-modified oligonucleotides to maleimide tips, (see manual [AFM\\_tip\\_with\\_maleimide](#)) unless some oligonucleotide is only available with an amino function.



**Figure 2:** Reaction of amino-functionalized AFM tips with Aldehyde-PEG-NHS and subsequent coupling of protein. Please, note that coupling is performed in sodium cyanoborohydride which converts the initially formed C=N bond into a stable C-N bond.

The experimental details for the **first step** are described in the manual *AFM\_tip\_aminofunctionalization*.

**For the second step**, the contents of one crimp-sealed vial of Aldehyde-PEG-NHS (3.3 mg) 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**). Alternatively, a graduated 10 mL measuring cylinder can be cut between the 1 and 2 mL marks and the rim smoothed with a hot flame. Triethylamine (30  $\mu$ L) 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  $\times$  10 min incubation) and dried with nitrogen gas. **We have never stored aldehyde-functionalized tips but immediately used them for coupling of proteins or other amine-containing molecules.**

**Before the third step**, a 1 M solution of sodium cyanoborohydride is freshly prepared by one out of two alternative methods.

- 13 mg sodium cyanoborohydride ( $\text{NaCNBH}_3 = \text{NaBH}_3\text{CN}$ ) is weighed into a screw-cap glass vial (2 mL). Exert due caution because of the high toxicity of this compound! **See last Note for the proper procedure!** Then, 20  $\mu$ L of 100 mM NaOH (4 mg/mL NaOH in water, see list of materials at the end of the manual) is added which ensures that no toxic HCN is being released. Subsequently, 180  $\mu$ L of water is added and the solution is carefully mixed with the pipette tip. Only 2  $\mu$ L of this solution is required in the fourth step, the rest should be diluted with water (e.g., 500 mL) containing >3 pellets of NaOH (or ~5 g of sodium carbonate) and subsequently flushed down the drain with copious amounts of water.
- Alternatively, if the crystals of sodium cyanoborohydride are large, it may be easier to work with 32 mg, adding 50  $\mu$ L of 100 mM NaOH and 450  $\mu$ L of water. Only 2  $\mu$ L of this solution is required in the fourth step, the rest should be diluted with water (e.g., 500 mL) containing >3 pellets of NaOH (or ~5 g of sodium carbonate) and subsequently flushed down the drain with copious amounts of water.
- **We do not recommend** using a stock solution of sodium cyanoborohydride (e.g., 5 M sodium cyanoborohydride, Sigma-Aldrich No. 296945) because its high sodium hydroxide content (1 M) makes proper pH adjustment during the coupling reaction extremely tricky. Moreover, the large quantity of this highly toxic solution poses a high safety risk.

**For the third step**, a piece of Parafilm is pressed onto the inner surface of a polystyrene Petri dish and several cantilevers are placed onto the Parafilm in a radial arrangement, with the tips in the center and facing upwards. The tipped ends must be so close together that one droplet of the protein solution (100  $\mu$ L) covers all tips at the same time. The typical concentration of antibodies in this reaction is 0.15-0.3 mg/mL but successful coupling has also been observed at 0.03 mg/mL. Subsequently, 2  $\mu$ L of the 1 M sodium cyanoborohydride solution is added and cautiously mixed into the droplet with the pipette. The Petri dish is covered with its lid and the reaction is allowed to proceed for one hour at room temperature. Subsequently, a 5  $\mu$ L aliquot of ethanolamine (1 M, pH 8.0, see list of materials at the end of the manual) is added

and cautiously mixed into the droplet with the pipette. After 10 min, the cantilevers are washed in PBS (3 × 5 min) and stored in a 24 well plate under PBS at 4°C for up to 1-2 weeks.

#### Notes:

- Phosphate, borate, and Hepes (or other Good buffers) are fully compatible with the aldehyde linker.
- The acetal/aldehyde linking method is not applicable if the protein sample contains buffer components with primary amines (e.g., **Tris** or **glycine**) or **ammonium** bicarbonate or **ammonium** acetate. In this case, the protein sample (50-100 µL) must be dialyzed, preferably with a "Side-A-Lyzer MINI Dialysis Device" from Pierce, with a molecular weight cut-off (MWCO) of 10K or 20K. Dialysis should be against 500 mL buffer for ~36 h, with several buffer changes.
- The aldehyde linking method does not work for proteins in presence of detergent. The reason is that detergent eliminates pre-adsorption of protein to the AFM tip which is indispensable in case of slow coupling reactions such as disulfide or aldehyde coupling [Kamruzzahan et al. (2007) Bioconjugate Chem. 17, 1473-1481].<sup>1</sup>
- In contrast, detergent is allowed if the concentration of the amine-containing ligand is  $\geq 5$  mM because then no pre-adsorption is required for fast coupling to the aldehyde functions.
- By analogy to the cyanhydrine reaction of cyanide (NaCN, KCN), **sodium azide** (**NaN<sub>3</sub>**) could theoretically react with the benzaldehyde function on the tip. The chances are very low that it actually happens because this reaction has never been reported in the literature. Moreover, even cyanide has a low tendency towards the cyanhydrine reaction under the conditions of AFM tip functionalization. In fact, low concentrations of cyanide are formed by hydrolysis of sodium cyanoborohydride, without any adverse effect on protein coupling. If you want to be on the safe side, you can remove azide by dialysis with a Slide-A-Lyzer (see first note).
- If antibodies are stabilized with an excess bovine serum albumin (BSA) or gelatin, then coupling to the aldehyde on the AFM tip is impossible because BSA has a much higher chance to get coupled. BSA can be removed from the sample by gel filtration on a Superdex 200 column (GE Healthcare). Alternatively, you can use a Melon gel kit from Thermo Scientific for removal of BSA or gelatin (<http://www.piercenet.com/files/TR0055-Melon-Gel-for-BSA.pdf>).
- By the same rationale, crude ascites fluid or serum cannot be used because only a minor fraction of the total protein content consists of the desired antibody.
- 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  $\mu\text{L}$  syringe is suggested for chloroform (part no. 81230, RN-type) and a 50  $\mu\text{L}$  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  $\times$ ). Then, the piston is removed and carefully rinsed with isopropanol. The barrel 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  $\times$ ).
  - 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 hands.

- Extreme caution is necessary when weighing the aliquots of sodium cyanoborohydride. The highest risk is breathing the dust and contact with the skin. Use a gas mask, a lab coat, and gloves for weighing. All material transfers must be performed in a well ventilated hood. Place the empty vial (2 mL) with the screw cap on the balance, press the re-zero button, carry the vial to the hood, add a tiny amount of  $\text{NaCNBH}_3$ , close the screw cap and put the closed vial onto the balance. Go back and forth between hood and balance and add/remove until the net weight of cyanoborohydride in the vial is 13 mg. Alternatively, you can place the balance into the hood and prepare many aliquots which are then stored in a desiccator in a poison cupboard. Clean the area in the hood with an alkaline solution (e.g., 1%  $\text{Na}_2\text{CO}_3$ ).

## Materials

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  $-20^\circ\text{C}$ .

- 140 mmol NaCl ( $58,44 \text{ g/mol} \times 0,140 \text{ mol} = \mathbf{8.182 \text{ g NaCl}}$ )
- 2.7 mmol KCl ( $74,56 \text{ g/mol} \times 0.0027 \text{ mol} = \mathbf{0.201 \text{ g KCl}}$ )
- 10 mmol  $\text{Na}_2\text{HPO}_4$  (in case of anhydrous dibasic sodium phosphate:  $141.96 \text{ g/mol} \times 0.010 \text{ mol} = \mathbf{1.420 \text{ g anhydrous Na}_2\text{HPO}_4$ ; in case of the heptahydrate:  $268.07 \text{ g/mol} \times 0.010 \text{ mol} = \mathbf{2.681 \text{ g Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ )
- 1.8 mmol  $\text{KH}_2\text{PO}_4$  ( $136.09 \text{ g/mol} \times 0.0018 \text{ mol} = \mathbf{0.245 \text{ g anhydrous KH}_2\text{PO}_4$ ).

100 mM NaOH is prepared by weighing 1 pellet of solid NaOH (~ 300 mg) into a beaker (100-250 mL) and adding water to give a final concentration of 4 mg/mL. As an example, 320 mg would be dissolved in  $320/4 = 80 \text{ mL}$  of water to give a 100 mM concentration. Aliquots (100  $\mu\text{L}$  or larger) are frozen and stored at  $-20^\circ\text{C}$ .

1 M ethanolamine (pH 8.0) is prepared by dissolving 975 mg ethanolamine hydrochloride in water at a final volume of 10 mL and adjusting the pH to 8.0 with 20% NaOH (towards the end with 2% NaOH). Aliquots (20  $\mu\text{L}$  and larger ones) are frozen and stored at  $-20^\circ\text{C}$  for up to several years.









### Literature focusing on aldehyde-functionalized AFM tips:

Ebner, A., Wildling, L., Kamruzzahan, A. S. M., Rankl, C., Wruss, J., Hahn, C. D., Hölzl, M., Kienberger, F., Blaas, D., Hinterdorfer, P., and Gruber, H. J. (2007) A new, simple method for linking of antibodies to atomic force microscopy tips. *Bioconjugate Chem.* 18, 1176-1184.

Wildling, L., Unterauer, B., Zhu, R., Rupprecht, A., Haselgrübler, T., Rankl, C., Ebner, A., Vater, D., Pollheimer, P., Pohl, E., Hinterdorfer, P., and Gruber, H. J. (2011) Linking of sensor molecules with amino groups to aminofunctionalized AFM tips. *Bioconjugate Chem.* 22, 1239-1248.

### Risk and Safety

	<b>Chloroform</b> (CHCl <sub>3</sub> ): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37
	<b>Diethyl ether</b> , 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)
	<b>Ethanolamine hydrochloride</b> , H315-H319-H335, P261-P305 + P351 + P338, R36/37/38, S26
	<b>Isopropanol = 2-propanol</b> : flammable, H225-H319-H336, P210-P261-P305 + P351 + P338, R11-36-67 (Europe), S7-16-24/25-26 (Europe)
	<b>Sodium cyanoborohydride</b> , NaCNBH <sub>3</sub> = NaBH <sub>3</sub> CN, contact with acids liberates very toxic gas (hydrogen cyanide, HCN), H228-H260-H300-H310-H314-H330-H410, P210-P223-P231 + P232-P260-P370 + P378-P422, R11-15-26/27/28-32-34-50/53 (Europe), S26-28-36/37/39-43-45-61
	<b>Triethylamine</b> (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.