Functionalization of AFM tips with tris-NTA for binding of His_6-tagged proteins

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2. IPR: Work involving tris-NTA is covered by patents (e.g., US 2008/0038750 A1). For academic research, please contact the inventor (Prof. Robert Tampé, Biocenter, Frankfurt am Main, Germany, tampe@em.uni-frankfurt.de). For commercial applications you have to obtain a license from the patent owner.
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4. Exclusion of warranty: The procedures described in this manual have successfully been applied by different users in our laboratory. We have done our best to provide descriptions that will enable reproduction in other laboratories. Nevertheless, failure may occur due to impurities or ingredients/components or circumstances which cannot be foreseen.
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 tris-NTA

short version

for risks and details see full length procedure

Please, note that work involving tris-NTA is covered by patents (e.g., US 2008/0038750 A1). See first page for possibilities to work with tris-NTA.

1. Aminofunctionalization of the cantilever(s) (see *02_AFM_tip_aminofunctionalization*).
2. Coupling of Maleimide-PEG-NHS (see *06_AFM_tip_with_maleimide*.)
3. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
4. Pre-mix the following components:
   - 50 µL disulfide-tris-NTA (1 mM) in water
   - 2 µL EDTA (50 mM, pH 7.5)
   - 5 µL Hepes (500 mM, pH 7.5)
   - 2 µL TCEP hydrochloride (50 mM)
   - 2.5 µL Hepes (500 mM, pH 9.6)
5. Pipet the mixture onto the cantilever(s), cover lid, incubate for 2-4 h
6. Wash in Hepes buffer or Tris buffer (3 × 5 min)
7. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
8. Pre-mix 100 µL His_6 protein with 4 µL $NiCl_2$ (5 mM)
9. Pipet the mixture onto the cantilever(s), cover lid, incubate for 1.5 h (0.5 µM protein) or 3 h (0.2 µM protein)
10. Wash in Hepes buffer or Tris buffer (3 × 5 min)
11. Mount cantilever in AFM setup

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Please, read the preceding manual *01_AFM_tip_functionalization_in_general* for the basic concept of AFM tip functionalization with long flexible PEG linkers.

Please, read the manual *02_AFM_tip_aminofunctionalization* for the details on how amino groups are generated on the AFM tip surface.

Please, read the manual *06_AFM_tip_with_maleimide* for coupling of Maleimide-PEG-NHS to the amino-functionalized tip surface.

Nowadays, most genetically engineered proteins are expressed with a hexahistidine tag (His_6). The reason is that His_6 tags are specifically bound by nickel affinity columns, as depicted in Figure 1 [1,3-5,11]. The affinity matrix carries nitrilotriacetate (NTA) groups which bind Ni^{2+} ions on the time scale of many hours without leaching [1]. Two coordination sites on the Ni^{2+} ion are still available for subsequent binding of two histidine residues [1]. In this way, the His_6-tagged protein is specifically retained on the chromatography column while all other proteins are washed away. Finally, the His_6-tagged protein can be released with imidazole or with EDTA (see Figure 1).

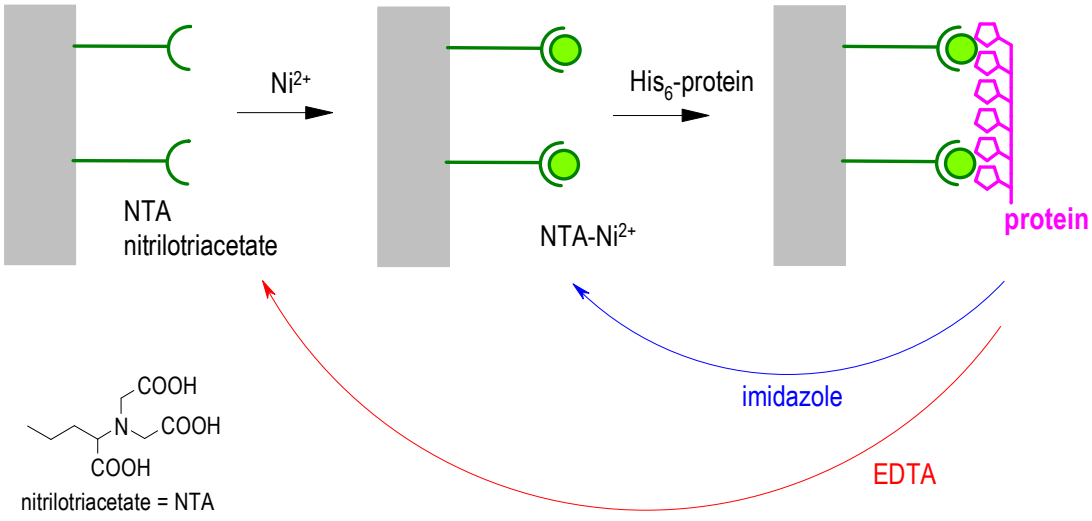

Figure 1: Immobilized metal ion affinity chromatography (IMAC): Surface-bound tris-NTA groups are loaded with Ni^{2+} ions for specific binding of His_6-tagged proteins [1,3-5,11]. Release can be triggered with imidazole or with EDTA.

The same principle was appealing for immobilization of His_6-tagged proteins as sensor molecules on biosensor surfaces [2]. However, in rigorous model experiments it was discovered that the bond between one immobile Ni^{2+} ion and two histidines was rather weak, with a dissociation half time in the order of 10 minutes [12,13]. By variation of the lateral

density of the NTA functions on the chip surface it was shown that high affinity (and long-lasting immobilization) was only achieved at high lateral density of the NTA functions [9,13,16,17]. From these experiments it became clear that the observed stable binding of His_6-tagged proteins on nickel columns was due to simultaneous binding one His_6 tag on at least two adjacent NTA-Ni^{2+} functions on the gel surface [9].

For obvious reasons, the maximal affinity will be reached if three NTA-Ni^{2+} groups are located in such close proximity that all six histidine residues of the His_6 tag are bound by the three Ni^{2+} ions. For this reason, several versions of "tris-NTA" have been synthesized which contain three NTA groups in rather close proximity [6-8,10,14,18]. One example is shown in Figure 2. It belongs to a series of similar tris-NTA molecules [8,10] which have been synthesized in the laboratory of Prof. Robert Tampé and Prof. Jacob Piehler. The disulfide form is good for storage. For coupling to maleimide groups, the thiol form must be generated by addition of TCEP. Fortunately, TCEP does not react with maleimides, therefore it can be added during the reaction of a thiol with a maleimide!!!

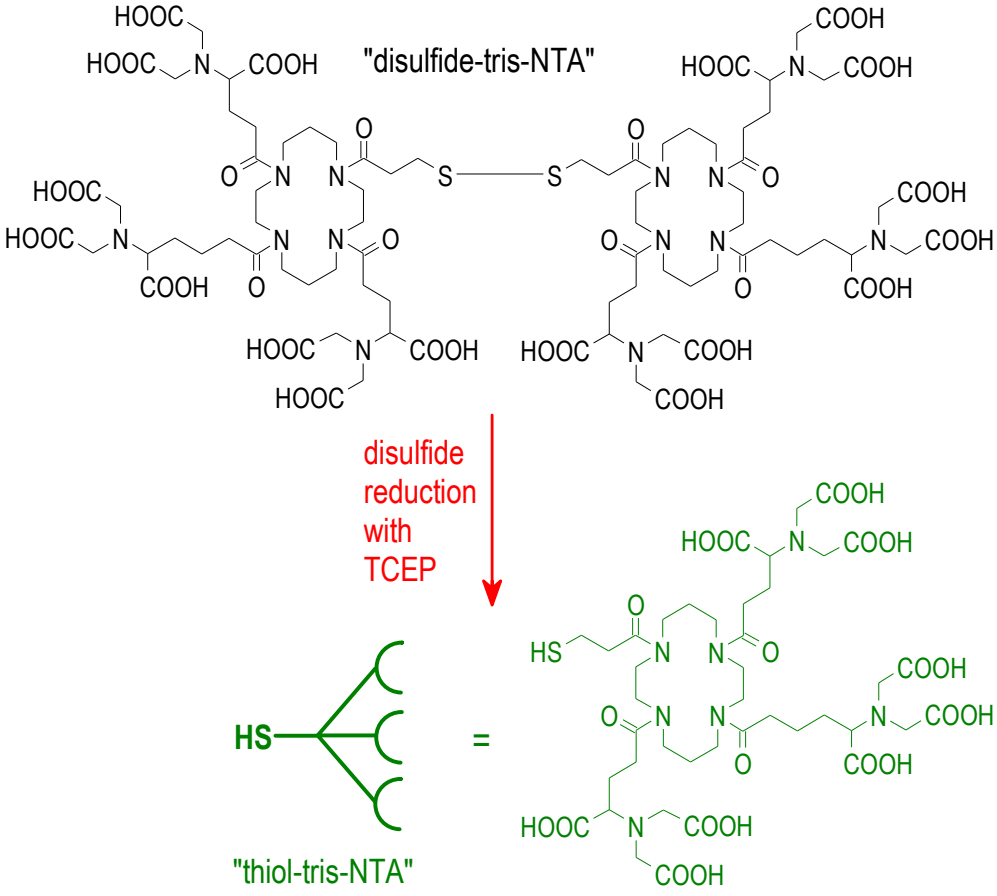

Figure 2: Structure of "disulfide-tris-NTA" and "thiol-tris-NTA". The disulfide is instantaneously cleaved into two thiol molecules by use of TCEP (at neutral pH). EDTA is required to prevent re-oxidation of thiol into disulfide by ambient air.

The application of tris-NTA for AFM tip functionalization is straightforward (Figure 3). The first two steps are the same as described in the manual *06_AFM_tip_with_maleimide*. In step No. 3, the AFM tip is simultaneously treated with disulfide-tris-NTA and TCEP (compare Figure 2), resulting in covalent binding of thiol-tris-NTA to maleimide. In step No. 4, the tip is loaded with $NiCl_2$, either before or during binding of His_6-tagged protein (see Figure 3).

We have already published AFM experiments with tris-NTA on the tip, except that the coupling scheme was inverted (disulfide on the PEG linker and maleimide on tris-NTA) [15].

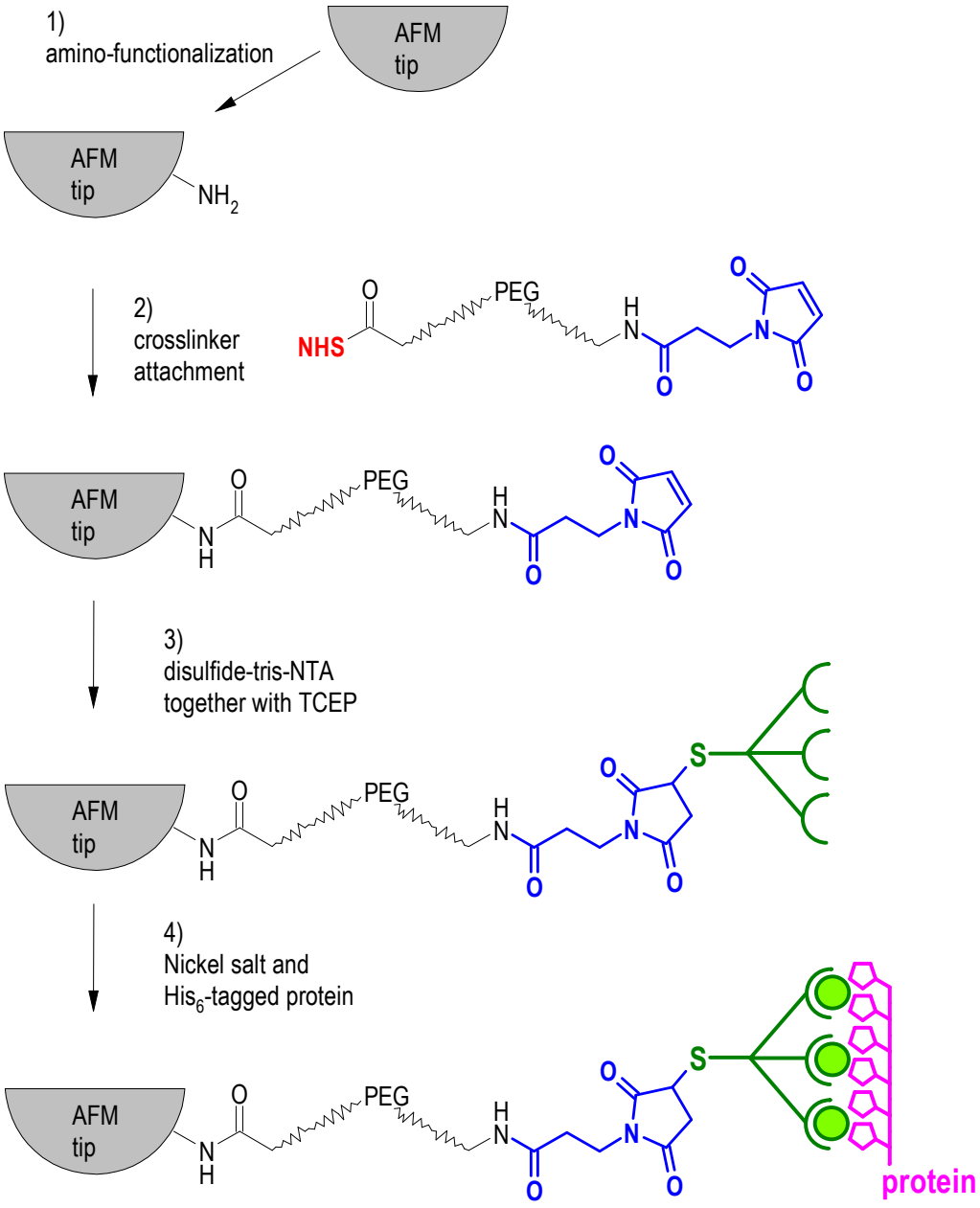

Figure 2: AFM tip functionalization with tris-NTA groups which are loaded with Ni^{2+} ions for subsequent binding of His_6-tagged proteins.

The experimental details for the **first step** are described in the manual *02_AFM_tip_aminofunctionalization*.

The **second step** (coupling of Maleimide-PEG-NHS) is described in the manual *06_AFM_tip_with_maleimide*.

Before the third step, stock solutions of EDTA (50 mM, pH 7.4), TCEP hydrochloride (50 mM) and Hepes (500 M, pH 7.5 and pH 9.6) are prepared, as described in the list of Materials (see below). Small aliquots can be stored at -20°C and thawed for daily use.

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 all of them can be wetted simultaneously when adding one 100 µl droplet of aqueous solution. This addition, however, is only done after sample mixing (see next paragraph).

Then, 50 µL of an aqueous solution of 1 mM disulfide-tris-NTA (see Materials) is pipetted into a reaction vial (1.5 mL or 2 mL) or a polystyrene tube (4 – 5 mL). 2 µL 50 mM EDTA (pH 7.5) is added and the sample is mixed with a 50 µL digital pipette. 5 µL of 500 mM Hepes (pH 7.5) is added and the sample is mixed. This step introduces high buffering capacity, as needed for the next steps. 2 µL of 50 mM TCEP hydrochloride is added and the sample is mixed. Hereby the pH drops below 7. Finally, 2.5 µL of 500 mM Hepes (pH 9.6) is added. Hereby the pH is raised to about 7.6.

Normally, the same volume of 50 mM TCEP hydrochloride and of 500 M Hepes (pH 9.6) must be added to adjust pH ~ 7.5. In this exceptional case, more of the basic Hepes solution is added to compensate for the six molecules of trifluoroacetic acid (TFA) which are bound on each disulfide-tris-NTA.

The optimal pH window is narrow: At pH ≤ 7 the kinetics of coupling will become rather slow. At pH ≥ 8 the maleimide group can be degraded by hydrolysis.

The sample mixture is transferred onto the cantilever tips, the Petri dish is covered with its lid, and the reaction is allowed to proceed at room temperature for 2-4 h. Subsequently, the cantilevers are washed in Hepes or Tris buffer (3 × 5 min). We have not tried to store the tips and used them immediately. If you want to store a tris-NTA tip, you should wash it with water (3 × 1 min), dry it with a stream of nitrogen gas and place it in an argon-filled desiccator. Theoretically, the tris-NTA groups on the tip should be stable for many weeks under argon but we have not tested this aspect.

The fourth step is again performed on a piece of Parafilm (as in step No. 3) which has been pressed onto the inner surface of a polystyrene Petri dish. The cantilever is placed on the Parafilm with the tipped end facing upwards. The His_6-tagged protein (100 µL, see Notes for the allowed buffer components) is mixed with 4 µL of a 5 mM $NiCl_2$ stock solution to achieve a final Ni^{2+} concentration of 200 µM. This sample is pipetted onto the cantilever tips, the Petri dish is covered with its lid, and the tip is incubated for 1.5 h (in case of 0.5 µM His_6 protein) or for 3 h (in case of 0.2 µM His_6 protein). The cantilever is washed in Tris or Hepes buffer (3 × 5 min) and mounted in the AFM setup.

Having performed force spectroscopy or TREC microscopy with this tip, you could try to regenerate it by removing the His_6 protein and attaching another His_6-tagged protein. For this purpose, the tip should be immersed in 500 mM imidazole (pH 7.4 adjusted with HCl) for 10

min and subsequently washed in the same solution (3 × 1 min). If the tip is to be re-used immediately, it should be washed with Hepes buffer (3 × 1 min). If it is to be stored, it should be washed with water (3 × 1 min), dried with a stream of nitrogen gas and stored in an argon-filled desiccator. By analogy to biosensor results it the regeneration should work well. We would be grateful if you report to us the results of such attempts.

Notes:

- The 1 mM concentration of disulfide-tris-NTA results in a 2 mM concentration of thiol-tris-NTA. By analogy to a small thiol derivative of biotin [Linda Wildling, Ph.D. thesis at Johannes Kepler University Linz], this concentration is high enough to ensure that all maleimides on the AFM tip react with thiol-tris-NTA within 2-4 h.
- The concentration of TCEP hydrochloride in the above reaction mixture is 2 mM which is sufficient to reduce 2 mM disulfide into 4 mM thiol component. An equal volume (2 µL) of 1 M Hepes (pH 9.6) would be required to counterbalance the acid introduced by addition of TCEP hydrochloride! However, the actual addition of 1 M Hepes (pH 9.6) is 2.5 µL because 1 mM disulfide-tris-NTA contains six trifluoroacetic acid molecules (TFA) which must also be neutralized by basic Hepes solution.
- The order of the pipetting steps is critical and must not be changed. For instance, if TCEP is mixed with Hepes (pH 9.6) in absence of sample, then TCEP may quickly oxidize and become inactive. The sequence of pipetting steps has been optimized to minimize the adverse effect of air on TCEP and the thiol components!
- EDTA is important (and sufficient) to prevent re-oxidation during the coupling of thiol-tris-NTA on a time scale of few hours. For longer reaction times it is advantageous to react under argon atmosphere, in addition.
- EDTA must not be contained in the sample of the His_6-tagged protein, otherwise the Ni^{2+} ions would get extracted from tris-NTA and no binding of His_6 protein will occur.
- Imidazol must also be absent in the sample of the His_6-tagged protein in order to allow for binding towards the tris-NTA-Ni^{2+} functions on the AFM tip.
- PBS (phosphate-buffered saline) is not compatible with Ni^{2+} ions because nickel phosphate is insoluble at neutral pH. Tris buffer or Hepes or any another "Good buffer" is fine.
- The pH of the His_6 protein sample should be above 7, otherwise the His_6-tagged protein will dissociate from the tris-NTA-Ni^{2+} functions on the AFM tip.
- 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.

Materials

50 mM EDTA is prepared by weighing 931 mg of disodium EDTA dihydrate (EDTA-Na_2.$2H_2O$) into a beaker (50 mL) and adding water to a final volume of ~40 mL. A stir bar is added and the suspension is stirred with dropwise addition of NaOH (initially 20% NaOH, towards the end 2% NaOH) to adjust a pH of 7.4. The solution is transferred into a volumetric flask (50 mL) or a graduated cylinder and water is added to give a final volume of 50 mL. Aliquots of different size (2 mL, 200 µL, 20 µL) are stored at -20°C for up to several years.

500 mM Hepes (pH 7.5) is prepared by dissolving Hepes (free acid) in water at a concentration of 119.2 g/L. As an example, 11.915 g Hepes is weighed into a 100 mL graduated cylinder and water is added to give a final volume of 80 mL. The suspension is mixed until the solid is completely dissolved. Then water is added to give a total volume of 100 mL and the solution is mixed again. Alternatively, a 1 M stock solution of Hepes acid can be purchased, e.g., for Sigma-Aldrich, and diluted with an equal volume of water to give 500 mM Hepes acid. Subsequently, the 500 mM stock solution is transferred to a beaker and stirred while slowly adding 20% NaOH (5 M) until the final pH reaches the value 7.5. At this point, the true Hepes concentration will be lower than 500 mM but this fact is ignored. Half of the total solution is immediately used for preparation of 500 mM Hepes (pH 9.6, see next paragraph) while afterwards the remainder of 500 mM Hepes (pH 7.5) is divided into aliquots of different size (2 mL, 200 µL, and 20 µL) and stored at -20°C for up to several years.

500 mM Hepes (pH 9.6) is prepared from half of the unused solution of 500 mM Hepes (pH 7.5, see previous paragraph) by continuing the addition of 20% NaOH until pH 9.6 has been reached. Aliquots of different size (2 mL, 200 µL, and 20 µL) are stored at -20°C for up to several years.

50 mM TCEP hydrochloride is prepared by weighing about 50 mg into a beaker and adding water to give a final concentration of 14.3 mg/mL. If the weight of the solid is exactly 50 mg then 3.49 mL of water is added. Aliquots of different size are prepared (e.g. 1 mL, 100 µL, and 10 µL) and stored at -20°C for up to several years. The high stability of TCEP in this stock solution is due to the low pH (~2) which prevents oxidation of TCEP in presence of air.

1 mM disulfide-tris-NTA is prepared by weighing about 5-10 mg into screw-cap glass vial and adding water to give a final concentration of 2.76 mg/mL. Please, note that the effective molecular weight of disulfide-tris-NTA is 2758 g/mol and not 2074.10 because the compound has been purified by reversed phase chromatography in presence of 0.1% trifluoroacetic acid (TFA). Six TFA molecules (114 g/mol) are bound to the six peripheral nitrogen atoms. The nitrogen in the center are not basic and do not bind TFA. Divide into 100 µL aliquots and store at -20°C for up to several years.

2 mM thiol-tris-NTA can be used in place of 1 mM disulfide-tris-NTA. The mass concentration should be the same (2.76 mg/ml) because two thiol molecules are equivalent to one disulfide and the molecular weight of the thiol form is almost exactly half of the disulfide form.

HBS (Hepes-buffered saline) is prepared by dissolving 8.766 g NaCl and 2.383 g Hepes (free acid) in water at a final volume of ~800 mL. Adjust the pH to 7.4, transfer the solution into a volumetric flask and add water to give a final volume of 1 L. The final concentrations of NaCl and Hepes are 150 mM and 10 mM, respectively. The solution must be mixed well. Aliquots (e.g., 100 mL) can be stored at -20°C for up to several years.

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Risk and Safety

Please, consult the Risk and Safety tables in the manuals for the first and second step of tip functionalization (*02_AFM_tip_aminofunctionalization and . 06_AFM_tip_with_maleimide).*

-	**EDTA disodium salt**, toxic if ingested or when the dust would be inhaled, not dangerous under normal working conditions
-	**Hepes, free acid,** irritant if ingested or when the dust would be inhaled, not dangerous under normal working conditions
	**Tris(carboxyethyl)phosphine hydrochloride** (TCEP hydrochloride): H314, P280-P305 + P351 + P338-P310, R34 (Europe), S26-27-36/37/39 (Europe)