Functionalization of AFM tips with Click Chemistry

Hermann J. Gruber, Institute of Biophysics, Johannes Kepler University,
Gruberstrasse 40, 4020 Linz, Austria – Europe

hermann.gruber@jku.at

Terms and conditions

1. If you publish data obtained with this manual, you are expected to cite the link from where it can be downloaded (http://www.jku.at/biophysics/content).
2. Our manuals are often updated. Only the newest version should be used. Please check whether you have the latest version. The date is obvious from the file name.
3. Copy right: You are entitled to distribute this manual, **provided that the document is not split or altered in any way**.
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 click chemistry

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 Azide-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 up to 5 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. The next step should be performed within 30 min. If the time between this step and the next two steps is longer than 30 min, then the tips should be stored under argon.
5. Prepare the reagent mixture for click chemistry in a Teflon reaction chamber which allows for constant removal of air by argon flushing. The solution should **additionally be bubbled** with argon **before** the addition of ascorbic acid and NaOH. The solution should constantly be superfused with slow stream argon gas which escapes between the chamber and the lid on top of the chamber (see sketch in **Figure 3**). It depends on the size of the reaction chamber, whether method A or B is used.

method A	**method B**	**ingredient**	**final concentration**
600 µL	300 µL	500 mM Tris/HCl (in water, pH 8.5)	300 mM Tris
180 µL	90 µL	DMSO	35.5 % DMSO in total
5 µL	2.5 µL	50 mM $CuSO_4$ (in water)	250 µM $CuSO_4$
25 µL	12.5 µL	10 mM co-catalyst No. 17 (in DMSO)	250 µM co-catalyst 17
150 µL	75 µL	6.5 mM **alkyne-ligand** (in DMSO)	1 mM alkyne
20 µL	10 µL	1 M ascorbic acid (in water)	20 mM ascorbate
20 µL	10 µL	1 M NaOH (in water)	20 mM NaOH
1 mL	0.5 mL	total volume	
16 mm	12 mm	inner diameter of reaction chamber (see **Figure 3**)	

6. Immerse up to 5 cantilevers in the reagent solution and incubate for ~17 h with constant protection by a slow stream of argon.
7. Wash cantilevers in PBS or any other buffer of choice (3 × 5 min).
8. Mount cantilever in AFM setup (or store in a 24 well plate under PBS at 4°C for 1-2 weeks).

hermann.gruber@jku.at

Functionalization of AFM tips with Click Chemistry

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 indicates the similarities of tip functionalization with maleimide and azide linkers.

- Both methods can be carried out in aqueous solution – but also in DMSO/water mixtures, in case that the ligand is not soluble in water.
- Both methods have in common that the reactive groups do not react with water on the time scale of tip functionalization. This means that all PEG linkers on the tip can be derivatized with ligand molecules before their reactivity is lost by hydrolysis.
- Both methods are rather efficient. This means that low concentrations of ligand molecules and short reaction times are sufficient for complete derivatization of the PEG linkers on the tip surface.

  In contrast, high concentrations of ligand molecules are required if the kinetics of coupling to the AFM tip is very slow. In this case, high concentrations of the ligand are required to functionalize all PEG linkers on the AFM tip with ligand molecules. A typical example is coupling ethylene diamine-ATP to aldehyde tips at 10 mM concentration [Wildling et al., 2011].

  As an exception, we can use low protein concentrations (≤1 µM) for coupling to aldehyde tips, in spite of the fact that the coupling of amines to aldehydes is rather slow [Hölzl et al., 2007]. The reason is that proteins are pre-adsorbed on the tip surface, reaching millimolar concentrations (!) in the vicinity of the tip-bound PEG linkers (see manual "acceleration_of_coupling_by_preadsorption") [Kamruzzahan et al., 2006].

- Both methods are very specific. This means that only the thiol on the ligand can react with the maleimide on the tip, and only the alkyne on the ligand can couple to the azide on the tip. As a consequence, the coupling site on the linker molecule is uniform and well defined.

The major advantage of click chemistry (**Figure 1B**) over thiol-maleimide coupling (**Figure 1A**) is the high stability of azide and alkyne groups. These two groups are rather unreactive, except when reacted with each other.

The high stability of azide and alkyne, plus their high reactivity in presence of Cu^{1+}, provide for quantitative attachment of multiple alkyne ligands to multivalent azide linkers [Bacharouche et al., 2015].

The major disadvantage of click chemistry is its need for Cu^{1+} ions as catalyst. Cu^{1+} can be generated *in situ* from Cu^{2+} with ascorbic acid but both ascorbate and Cu^{1+} are easily oxidized by ambient air (**Figure 4**). It is therefore mandatory to perform click chemistry in a special reaction chamber which is constantly perfused with argon gas to keep away the oxygen of ambient air.

No oxidation problem is encountered with "strain-promoted click chemistry" (**Figure 7**). Such "copper-free click chemistry" can be performed without argon, under ambient conditions. The disadvantage is the high hydrophobicity (poor water solubility) of the cyclooctyne derivatives.

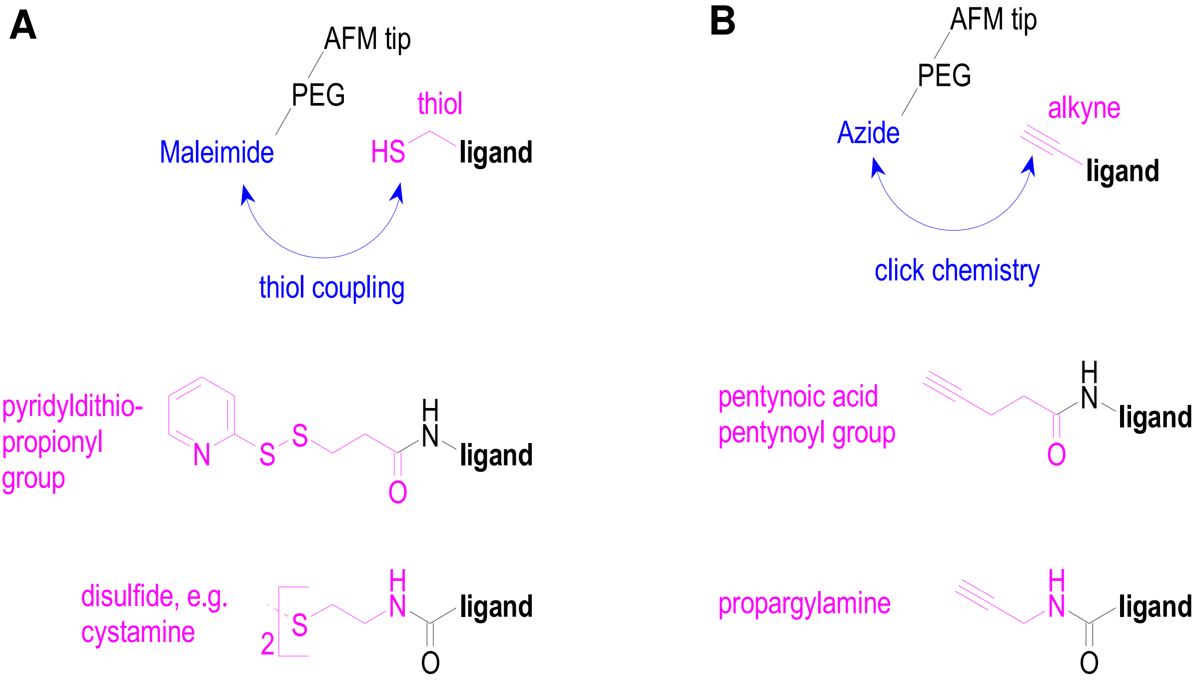

Figure 1: Comparison of AFM tips functionalization via (**A**) thiol coupling to maleimide linkers and (**B**) so-called "click chemistry" where C≡C bonds (alkynes) are coupled to azide groups. (**ad A**) Some biomolecules have endogeneous thiol groups; examples are some natural proteins (e.g. BSA) or cysteine mutants or synthetic oligonucleotides. Alternatively, ligand molecules can be derivatized with pyridyldithiopropionyl groups or with cystamine and then converted into free thiols by addition of a reducing agent (TCEP). (**ad B**) Biomolecules do not contain C≡C bonds (alkynes). The only exception are genetically engineered proteins containing a nonnatural amino acid. However, biomolecules or synthetic ligands can be derivatized with pentynoic acid or with propargylamine which contain alkyne groups (–C≡CH).

Azide-PEG18-NHS

Figure 2: Structure of the Azide-PEG18-NHS which is used to couple alkyne-functionalized ligands to amino-functionalized AFM tips (see **Figure 3**).

hermann.gruber@jku.at

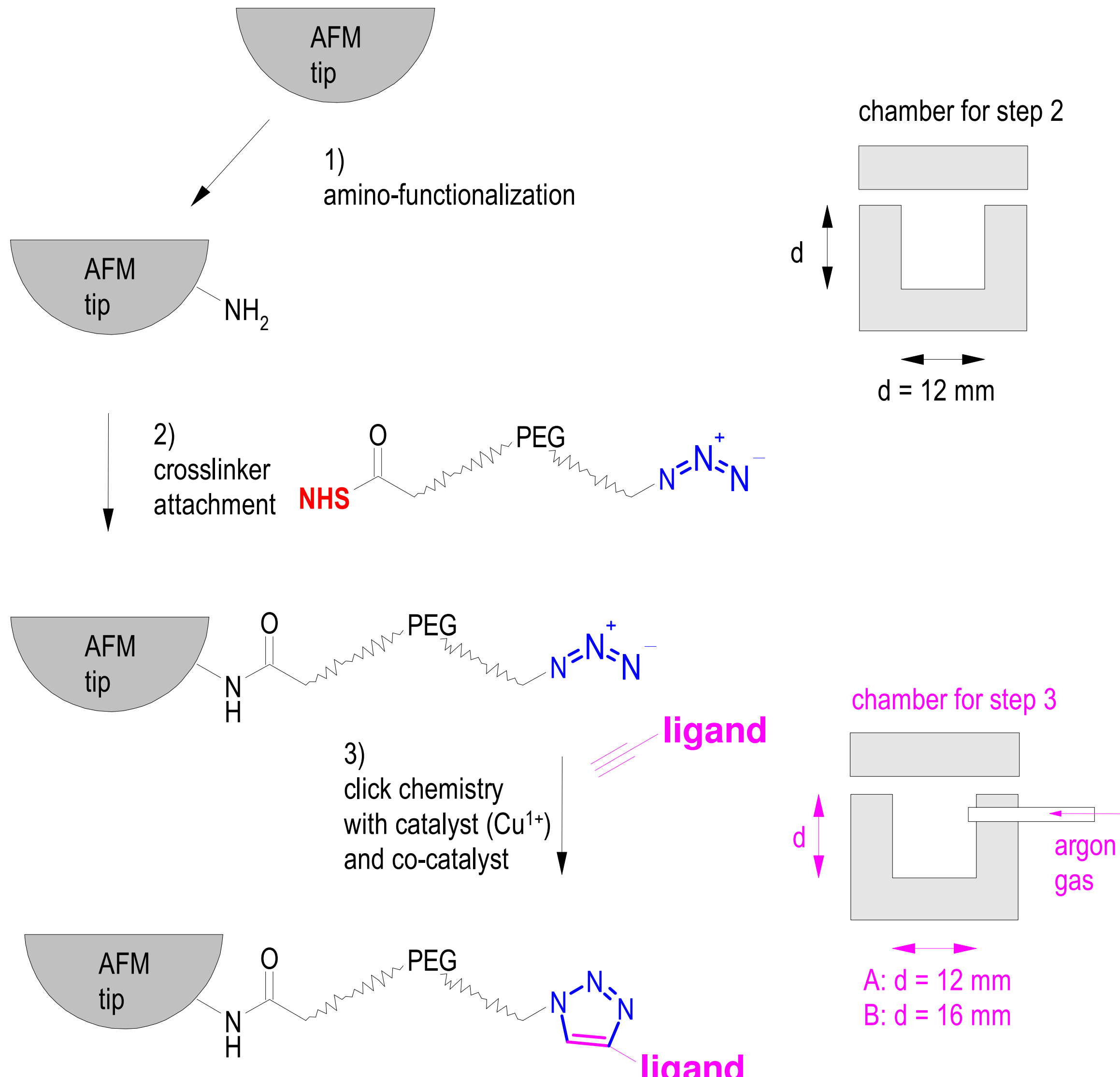

Figure 3: Reaction of amino-functionalized AFM tips with Azide-PEG-NHS and subsequent coupling of an alkyne-derivatized ligand via click chemistry. Please, note that coupling is performed with the help of a catalyst (Cu^{2+} and ascorbate) and accelerated by a co-catalyst. Step 2 should be performed in the common reaction chamber (upper sketch): Step 2 should not be performed in the argon chamber with d = 12 mm (lower sketch) unless the argon gas is turned off (argon would blow off the solvent and triethylamine). Step 3 must be performed in a chamber with an argon inlet (lower sketch). The lid should allow for slow escape of argon during perfusion.

A protocol for click-coupling of antibodies to gold-coated AFM tips has recently been published [Chen et al., 2009]. In **Figure 3**, we present a method for click chemistry silicon or silicon nitride cantilevers. The first step 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 (Azide-PEG-NHS, see **Figure 2**), and the third step is coupling of the alkyne-ligand by

click chemistry. The protocol has been used in two studies from our laboratory [Zhu et al., 2016; Köhler et al., manuscript in preparation].

Figure 4: In-situ preparation of **Cu^{1+}** by reaction of Cu^{2+} ions with ascorbic acid. **Cu^{1+}** is required as catalyst for the click reaction of azide and alkyne (see **Figure 3**).

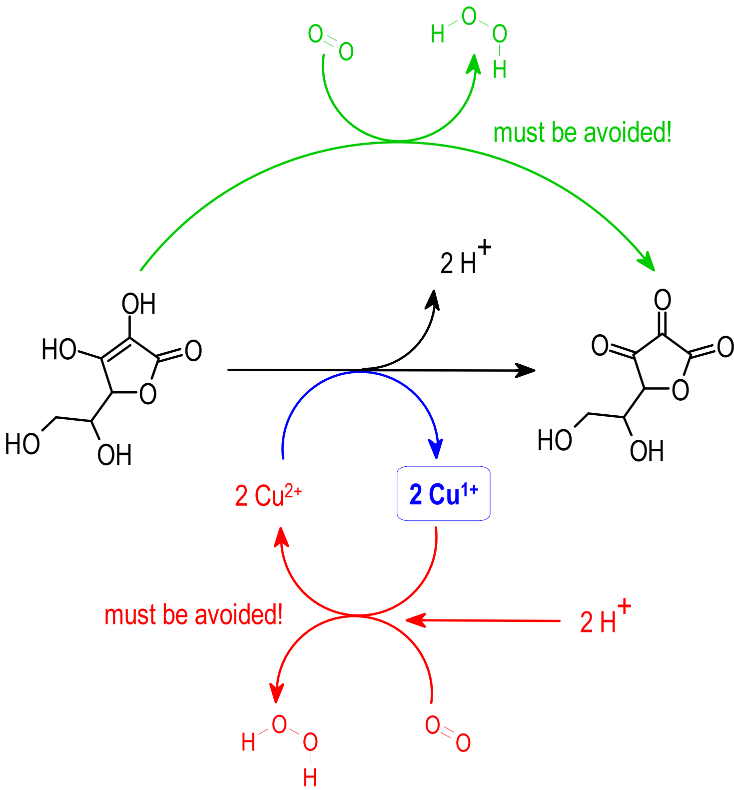

Figure 5: Undesired side reactions caused by air: The generation of **Cu^{1+}** and the whole click reaction must be performed under an argon atmosphere, for two reasons: (1) **Cu^{1+}** would quickly be re-oxidized into Cu^{2+} by the oxygen content of air. (2) Ascorbic acid will also quickly be oxidized by the oxygen content of air. Fortunately, the oxidation of ascorbic acid takes place at neutral pH only, not in the 1 M stock solution of ascorbic acid which is strongly acidic.

Copper-catalyzed click chemistry has the big advantage that the reacting groups are very simple, and very unreactive in absence of copper. It is easy to introduce simple alkyne groups in the ligand molecules (compare **Figure 1**). The downside of copper-catalyzed click chemistry is its high sensitivity to ambient air. As explained in the legend to **Figure 5**, it is essential to perform the reaction under argon atmosphere. Nitrogen gas cannot be used because it is lighter than air, thus nitrogen will quickly be replaced by air at the earliest occasion. – The only exception is working in a glove box which contains a slight superpressure of nitrogen, and a vestibule where the materials and tools can be placed and the air can be exchanged for nitrogen, before materials and tools are transferred to the main chamber of the glove box.

It should be noted that copper-catalyzed click chemistry is not very fast. Fortunately it can be accelerated when using a co-catalyst (**Figure 6**). The co-catalyst is commercially available (see legend to **Figure 5**) and its use is very convenient (see below procedure).

Figure 6: Structure of the "co-catalyst No. 17" [Lewis et al., 2004] which is used to accelerate click chemistry reactions. The full name is bathophenanthrolinedisulfonic acid disodium salt hydrate (e.g.; product No. 146617 from Sigma-Aldrich). The two nitrogen atoms (**N**) coordinate and stabilize **Cu^{1+}** ions [Lewis et al., 2004]. This compound is very soluble in aqueous buffer, in DMSO, and in buffer/DMSO mixtures.

The problem of air sensitivity is eliminated when using "copper-free click chemistry". The latter method is also called "strain-promoted click chemistry". The principle is explained in **Figure 7**. A simple alkyne has the linear structure shown in (**A**). Here the C≡C bond and the two adjacent C-atoms lie on a straight line and experience no steric stress.

The alkynes (**B**) and (**C**) contain the triple bond in cyclooctyne rings where the structure C–C≡C–C is necessarily bent, due to the ring structure. Here, the triple bond has a high reactivity for azides, even in absence of copper catalyst. The reason is that coupling to the azide converts the triple bond into a double bond which is no longer strained by the ring system. The dibenzocyclooctyne (**C**) is more reactive than the simple cyclooctyne (**B**)

because in **C** the steric strain is accentuated by the two benzene rings and by the amide structure in the 8-membered ring.

The disadvantage of cyclooctyne derivatives is their poor solubility in water. This problem can only be compensated if the ligand molecule is very hydrophilic.

Figure 7: Comparison of copper-dependent ligands (**A**) with copper-independent click ligands (**B, C**). Copper-free click chemistry can only be performed with sterically strained alkynes, such as cyclooctyne derivatives. Here the coupling of the azide serves to relax the strain by converting the C≡C triple bond in (**B**) into a C=C double bond. The dibenzocyclooctyne derivative shown in green (**C**) is particularly reactive because the two benzene rings plus the amide group in the 8-membered ring maximize the steric strain in the cyclooctyne ring.

hermann.gruber@jku.at

Procedure

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 Azide-PEG-NHS (1 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 3**). 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. Up to five amino-functionalized cantilevers 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 azide-functionalized tips can be stored for several days under argon atmosphere.

Before the third step, it is first necessary to prepare the argon-perfused reaction chamber (see sketch in **Figure 3**, lower right) and to perform a test run with 0.5 mL of water in the small chamber A (or 1 mL water in the large chamber B). The chamber is chamber can be prepared by drilling a circular hole with a perfectly flat bottom into a small Teflon block (see sketch in **Figure 3**). A hole must be drilled into the wall of the chamber, close to the top, and an argon inlet must be installed. One option would be a hypodermic needle with a large diameter which tightly fits into the drilled hole. The barrel of a 1 mL disposable syringe can is cut to remove the "ears", so that the silicone tubing from the argon gas tank can be attached to the end of the syringe barrel. It is important to mount the Teflon chamber firmly on the base plate of a heavy ring stand with a clamp, otherwise the weight of the silicone tubing would render the chamber unstable.

For the third step, all ingredients except for ascorbic acid and NaOH (!!!) are mixed inside the reaction chamber (see Table 1, below). Then, the solution is bubbled with a stream of argon from a Pasteur pipette for 2 min.

Subsequently, the small aliquots of ascorbic acid and NaOH are added and bubbling with argon gas is repeated for 30 sec.

Then the argon line is connected to the argon inlet of the chamber and the argon flow is turned to a high level.

The cantilevers are immersed, the lid is placed on top (with a thin piece of Parafilm on one side between lid and chamber to allow for escape of argon) and the chamber is perfused with a high argon stream for another 5 min.

Then the argon flow is reduced to a minimum. Hereby it is important to adjust a constant pressure which is rather low – but not so low that it could accidentally drop to zero. At the same time, the needle valve should be opened very little – but not so little that the flow could stop spontaneously overnight.

After the click chemistry step, the cantilevers are transferred into PBS or any other buffer of choice and washed by incubation (3 × 5 min).

hermann.gruber@jku.at

The cantilevers can be stored in a 24 well plate under PBS at 4°C for up to 2 weeks before being mounted in an AFM setup.

Table 1:

method A	method B	ingredient	final concentration
600 µL	300 µL	500 mM Tris/HCl (in water, pH 8.5)	300 mM Tris
180 µL	90 µL	DMSO	35.5 % DMSO in total
5 µL	2.5 µL	50 mM $CuSO_4$ (in water)	250 µM $CuSO_4$
25 µL	12.5 µL	10 mM co-catalyst No. 17 (in DMSO)	250 µM co-catalyst 17
150 µL	75 µL	6.5 mM **alkyne-ligand** (in DMSO)	1 mM alkyne
At this stage, the solution is mixed by bubbling with argon gas for 2 min from a Pasteur pipette.			
20 µL	10 µL	1 M ascorbic acid (in water)	20 mM ascorbate
20 µL	10 µL	1 M NaOH (in water)	20 mM NaOH
1 mL	0.5 mL	total volume	
16 mm	12 mm	inner diameter of reaction chamber (see **Figure 3**)	
Immediately after addition of ascorbate and NaOH, the solution is again bubbled with argon gas from a Pasteur pipette for 30 sec. Then the argon gas line is attached to the argon inlet of the reaction chamber and the argon flow is turned on. The cantilevers are immersed in the solution with a tweezer, the chamber is covered with a lid and the chamber is perfused with a relatively high argon stream for 5 min. Subsequently, the argon stream can be turned down to a minimum level which still ensures that no air can flow into the reaction chamber. This minute argon flow is maintained overnight for about 16 to 18 hours.			

Notes:

- We decided to dissolve the alkyne-ligand and the co-catalyst No. 17 in DMSO because our alkyne ligands were not well soluble in water [Zhu et al., 2016]. Co-catalyst No. 17 is also very well soluble in pure water [Lewis et al., 2004]. If the alkyne ligand is well soluble in water, than water can be used as solvent for all reagents listed in Table 1!
- Please, note that a special reaction chamber is required to perform click chemistry (step 3) under rigorous exclusion of air. Two different chamber sizes are suggested for this purpose (see **Figure 3**), depending on the preference of the user. The smaller chamber (**A**) requires 0.5 mL reaction volume and the larger chamber (**B**) 1.0 mL. The larger one is easier to handle.
- If the lid of the argon-perfused reaction chamber has a perfect fit, then perfusion with argon will lead to vibration of the lid on the chamber. This can be prevented by inserting a small piece of Parafilm between chamber and lid on one side.
- Please, note: In the absence of ascorbate and NaOH the mixture is not yet sensitive to air. Nevertheless, this mixture must be bubbled with argon gas for 2 min, because it is necessary to expel all oxygen from the solution before the ascorbic acid is added.
- Interestingly, the 1 M stock solution of ascorbic acid is also not sensitive to the oxygen of air, for two reasons: (i) Ascorbic acid is strongly acidic and at low pH the ascorbic acid is not easily oxidized. (ii) The concentration of the stock solution is 1 M, while the concentration of oxygen in air is only ~6 mM (0.2g/L × 1mol/32g = 0.006 mol/L).

  However, when the ascorbic acid is diluted into the reagent mixture, then the ascorbic acid is diluted to a low concentration (20 mM) and at the pH of the Tris buffer is around 8 where ascorbic acid can rapidly be oxidized if the solution contains dissolved oxygen (we have seen complete oxidation of ascorbate within a minute at neutral pH when the solution was stirred in presence of air). Addition of NaOH will cause a further increase of the pH towards 8.5 and further accelerate the oxidation of ascorbate.

  For these reasons, it is necessary to bubble the mixture with argon gas, both before and after the addition of 1 M ascorbic acid and of 1 M NaOH.
- 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 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 or argon 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 gas 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.

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°C.

- 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**).

Tris buffer (500 mM, pH 8.5, MW = 121.14) is prepared by dissolving 1.211 g Tris base in about 80 ml water in an Erlenmeyer flask. The pH is adjusted to 8.5 by drop-wise addition of concentrated HCl with stirring. The expected consumption of concentrated HCl (37%, 12 M) for adjustment of pH 8.5 is between 1.0 and 1.5 mL. It is advisable to switch to from concentrated to dilute HCl when coming close to pH 8.5. The solution is transferred into a 100 mL graduated cylinder or measuring flask. The Erlenmeyer is rinsed with small quantities of water (few mL) and this water is added to the Tris buffer. This process is repeated until the total volume of the Tris buffer is exactly 100 mL. Then the Tris buffer is thoroughly mixed and divided into aliquots which are stored at -25°C. The aliquot size for daily use should be >600 µL or >300 µL (see Table 1). It is not convenient to divide the total quantity of 100 mL buffer into such small aliquots. Part of the solution should be divided into 10 mL aliquots in 15 mL Falcon tubes and stored at -25°C. When all small aliquots are consumed, one 10 mL aliquot is thawed, divided into the small aliquots and frozen again. Re-freezing is a problem with large biomolecules which would denature – but not with simple buffer solutions!

$CuSO_4$ stock solution (50 mM). The most common form is the pentahydrate ($CuSO_4 \cdot 5H_2O$, 249.69 g/mol). When using the pentahydrate, the 50 mM solution has a w/v-concentration of 12.48 mg/mL. For preparation of the 50 mM stock solution, an amount of about 200 mg $CuSO_4 \cdot 5H_2O$ is weighed into measuring cylinder and the exact "weight" (in mg) is recorded. Then, the exact "weight" (in mg) is divided by 12.48 mg/mL, yielding the desired volume (in mL) which gives a concentration of 50 mM $CuSO_4$. For example, if the "weight" of $CuSO_4 \cdot 5H_2O$ in the measuring cylinder is 207.3 mg, then 207.3 mg / 12.48 mg/mL = 16.6 mL total volume of the solution. Water is added to reach this final volume and the solution is carefully mixed. The stock solution is divided into aliquots and stored frozen. Although only 5 µL aliquots are needed for one experiment, the aliquot size should not be less than 100 µL because the temperature variations in a freezer could cause loss of water during prolonged storage when the volume is too small. For convenience, a part of the solution should first be divided into 1 mL aliquots and subsequently be divided into smaller aliquots when needed.

Co-catalyst 17 (10 mM) in DMSO. The full name is bathophenanthrolinedisulfonic acid disodium salt hydrate (MW = 590.52). The material can be obtained from Sigma-Aldrich (product 146617). The 10 mM concentration of the stock solution corresponds to a w/v-concentration of 5.90 mg/mL. An amount of about 80 mg is weighed into a 25 mL measuring cylinder and the exact "weight" (in mg) is recorded. Then, the exact "weight" (in mg) is divided by 5.90 mg/mL, yielding the desired volume (in mL) which gives a concentration of 10 mM co-catalyst 17. For example, if the "weight" of co-catalyst 17 in the measuring cylinder is 82.5 mg, then 82.5 mg / 5.90 mg/mL = 14.0 mL total volume of the solution. DMSO is added to reach this final volume and the solution is carefully mixed. This 10 mM stock solution is divided into 100 µL and 1 mL aliquots, as described for 50 mM $CuSO_4$ (see previous paragraph). Theoretically it would also be possible to dissolve this co-catalyst No. 17 in water. It is well soluble! We used DMSO because our alkyne ligands were not well soluble in water. For the same reason, it was advisable to reach a final concentration of 37% DMSO in the reaction mixture (see Table 1).

Alkyne ligand stock solution (6.5 mM) in DMSO. In our study, we chose a concentration of 6.5 mM [Zhu et al., 2016]. Theoretically, the reaction should also work at 10-fold lower concentrations [Lewis et al., 2004]. It is possible to prepare a DMSO stock solution and to freeze it at -25°C. Please, note that DMSO has a melting point of 15°C and that its boiling point is 189°C. Because of the low vapor pressure it is possible to store very small aliquots in the freezer, without loss of solvent by evaporation. If your alkyne ligand is well soluble in water, then you can also prepare a stock solution in water, rather than in DMSO. In this case, it is possible to switch from DMSO to water in all reagents listed in Table 1 (see Notes).

Ascorbic acid (1 M) in water. A 1 M stock solution of ascorbic acid (MW = 176.12 g/mol) has a w/v-concentration of 176.12 mg/mL. Roughly 2000 mg of ascorbic acid is weighed into a graduated 25 mL cylinder and the exact "weight" (in mg) is recorded. Then, the exact "weight" (in mg) is divided by 176.12 mg/mL, yielding the desired volume (in mL) which yields 1 M ascorbic acid. For example, if the "weight" of ascorbid acid in the measuring cylinder is 2072 mg, then 2072 mg / 176.12 mg/mL = 11.8 mL total volume of the solution. Water is added to reach this final volume and the solution is carefully mixed. This 1 M ascorbic acid solution is divided into 100 µL and 1 mL aliquots and stored at -25°C, as described for 50 mM $CuSO_4$ (see above). The ascorbic acid should be stable for years at -25°C because its pH is very low and its concentration is high (see Notes).

NaOH (1 M) in water. A 1 M stock solution of NaOH (MW = 40 g/mol) has a w/v-concentration of 40 mg/mL. One pellet of NaOH amounts to ~300 mg. Two pellets of NaOH are weighed into a 25 mL graduated cylinder and the exact "weight" (in mg) is recorded. Then, the exact "weight" (in mg) is divided by 40 mg/mL, yielding the desired volume (in mL) which gives a concentration of 1 M NaOH. For example, if the "weight" of NaOH in the measuring cylinder is 682 mg, then 682 mg / 40 mg/mL = 17.1 mL total volume of the solution. Water is added to reach this final volume and the solution is carefully mixed. This 1 M NaOH solution is divided into 100 µL and 1 mL aliquots and stored at -25°C, as described for 50 mM $CuSO_4$ (see above).

hermann.gruber@jku.at

Literature references:

Bacharouche, J., Degardin, M., Jierry, L., Carteret, C., Lavalle, P., Hemmerlé, J., Senger, B., Auzély-Velty, R., Boulmedais, F., Boturyn, D., Coche-Guérente, L., Schaaf, P., and Francius, G. (2015) Multivalency: influence of the residence time and the retraction rate on rupture forces measured by AFM. *J. Mater. Chem. B 3*, 1801-1812.

Chen, G., Ning, X., Park, B., Boons, G.-J., and Xu, B. (2009) Simple, Clickable Protocol for Atomic Force Microscopy Tip Modification and Its Application for Trace Ricin Detection by Recognition Imaging. *Langmuir 25*, 2860-2864.

Hölzl, M. Tinazli, T., Leitner, C., Hahn, C. D., Lackner, B., Tampé, R., and Gruber, H. J. (2007) Protein-resistant self-assembled monolayers on gold with latent aldehyde functions. *Langmuir 23*, 5571-5577.

Kamruzzahan, A. S. M., Ebner, A., Wildling, L., Kienberger, F., Riener, C. K., Hahn, C. D., Pollheimer, P. D., Winklehner, P., Hölzl, M., Lackner, B., Schörkl, D. M., Hinterdorfer, P., and Gruber, H. J. (2006) Antibody linking to atomic force microscope tips via disulfide bond formation. *Bioconjugate Chem. 17*, 1473-1481.

Lewis, W. G., Magallon, R. G., Fokin, V. V., and Finn, M. G. (2004) Discovery and characterization of catalysts for azide-alkyne cycloaddition by fluorescence quenching. *J. Am. Chem. Soc. 126*, 9152-9153.

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.

Zhu, R., Sinwel, D. Hasenhuetl, P. S., Saha, K., Kumar, V., Zhang, P., Rankl, C., Holy, M., Sucic, S., Kudlacek, O., Karner, A., Sandtner, W., Stockner, T., Gruber, H. J., Freissmuth, M., Newman, A. H., Sitte, H. H., and Hinterdorfer, P. (2016) Nanopharmacological force sensing reveals allosteric coupling in transporter binding sites. *Angew. Chemie, Int. Ed. Engl. 55*, 1719-22.

Risk and Safety

-	**L-ascorbic acid,** = vitamine C, not problematic on at the scale used here
-	**Bathophenanthrolinedisulfonic acid disodium salt trihydrate**, CAS No. 52746-49-3. No data are available concerning risk and safety. The material must be handled with great caution! Do not breath dust! Do not ingest! Avoid skin contact!

hermann.gruber@jku.at

	Chloroform ($CHCl_3$): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37. Do not inhale dust! Do not ingest! Avoid skin contact!
	Copper (II) sulfate, $CuSO_4$, H302-H315-H319-H410, P273-P305 + P351 + P338-P501. Do not inhale dust! Do not ingest! Avoid skin contact!
	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). Do not inhale! Do not ingest! Avoid skin contact!
-	**DMSO**, low toxicity solvent (LD50 Oral-Rat = 14.5 g/kg) and low vapor pressure (0.56 mbar at 20°C). Avoid skin contact! Do not ingest!
	Ethanolamine hydrochloride, H315-H319-H335, P261-P305 + P351 + P338, R36/37/38, S26. Do not inhale dust! Do not ingest! Avoid skin contact!
	Hydrochloric acid, concentrated HCl (37% = 12 M), H290-H314-H335, P261-P280-P305 + P351 + P338-P310. Extremely corrosive. Severe irritant. Extensive inhalation can cause fatal lung edema after several hours lag time (quick treatment with cortisone spray and cortisone can reduce the risk of a fatal edema). Handle in well ventilated hood. Very small quantities can shortly be handled outside a hood but evaporation of the concentrated stock solution must be kept at a minimum and the area must be well ventilated. The optimal gas mask filter type is E (yellow color code). Usually the filters cover several problems at once (e.g., ABEK for organic vapors, inorganic toxic gases, strong acids, and ammonia)
	Isopropanol = 2-propanol: flammable, H225-H319-H336, P210-P261-P305 + P351 + P338, R11-36-67 (Europe), S7-16-24/25-26 (Europe)
	Sodium hydroxide, NaOH, H290-H314, P280-P305 + P351 + P338-P310. Severely corrosive and irritant! Warning: When the pellets are dissolved in water, the solution becomes very hot and releases a very corrosive aerosol. For this reason, the pellets should be dissolved in a well ventilated hood. The concentrated stock solution (e.g. 20% w/v = 5 M) is very corrosive. Avoid skin contact! Do not sonicate solutions which contain NaOH because the aerosol is very dangerous when inhaled into the lung.

hermann.gruber@jku.at

	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
-	**Tris base** (= tris(hydroxymethyl)aminomethane) = Trizma® base. CAS No. 77-86-1. Short term contact causes no irritation of skin but may irritate the eye. Do not ingest! Do not inhale dust!

Be careful when using Pasteur pipettes which are connected to a nitrogen or argon 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 gas flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.

hermann.gruber@jku.at