Enzymatic Quantification of Biotin-PEG on Silicon Nitride Chips

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

hermann.gruber@jku.at

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

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

Please, carefully read the manual *AFM_tip_with_biotin* about the reaction of aminofunctionalized tips with Biotin-PEG-NHS.

AFM tip functionalization with linkers and proteins is performed in a sequence of many steps. Although some chemical expertise is required, many physicists, biologists, and medical scientists usually learned the methods within 1-2 weeks, either as guests in our laboratory, or by use of our written protocols.

In case of problems, we recommend to take resort to the avidin-biotin test system. The details of the avidin-biotin interaction are described in the introduction of the manual "*AFM_tip_with_biotin*". The avidin-biotin system has the following advantages:

- It does not require coupling of a protein to the AFM tip.
- The success of aminofunctionalization and linker attachment can be quantified.
- Quantification is done with two alternative methods:
  - AFM
  - or a macroscopic enzyme assay.

Figure 1A illustrates the attachment of biotin groups to AFM tips via long, flexible PEG chains. The presence of biotin groups on the AFM tip can be verified by force spectroscopy experiments on a dense layer of avidin molecules.

Figure 1B illustrates testing of the AFM tip by recognition force spectroscopy. The avidin test sample is prepared within minutes by adsorbing avidin to freshly cleaved mica. In force spectroscopy experiments, about 10-25% of all force-distance cycles will show rupture events with a characteristic unbinding force (~40 to 50 pN at typical loading rates). Specific interactions can be discriminated from unspecific adhesion events by performing parallel experiments in which free biotin (0.1 mg/mL = 0.4 mM) is included in the buffer [Riener et al., 2003]. – The experimental details of this AFM testing method are described in the manual "*AFM_tip_with_biotin*".

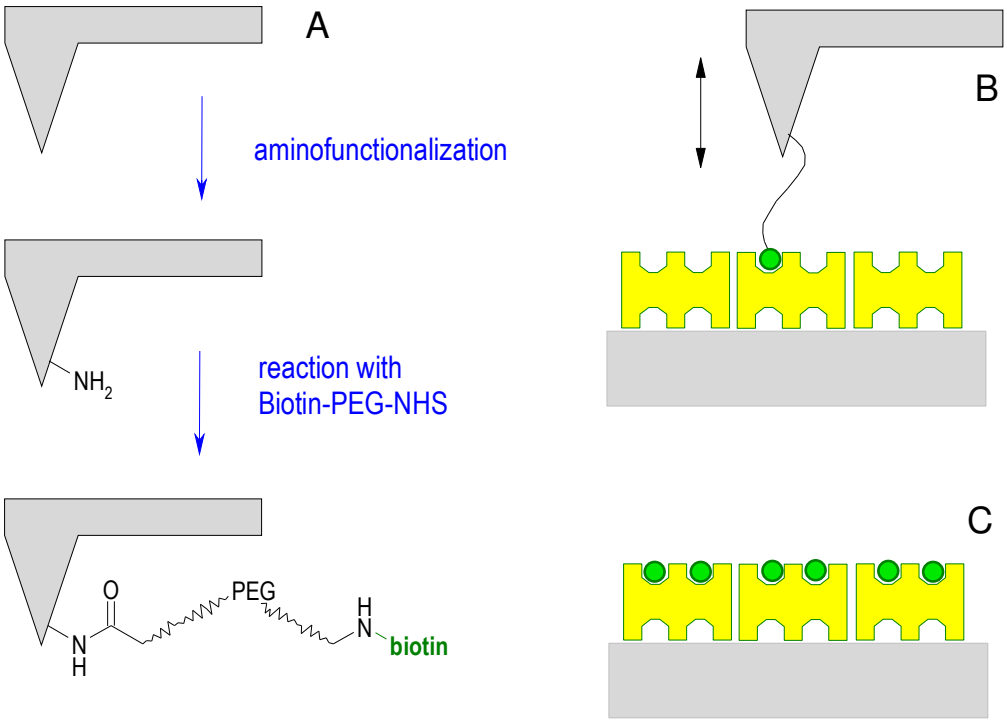

Figure 1: Test system for recognition force spectroscopy. (**A**) Preparation of AFM tips with biotin groups on PEG chains involves (i) aminofunctionalization of the cantilever, and (ii) attachment of Biotin-PEG-NHS to the amino groups. (**B**) Recognition force spectroscopy of the specific interaction between tip-bound biotin (**green sphere**) and mica-bound avidin shows a typical unbinding force of ~40 pN at a loading rate of 1.4 nN/s. Specific binding/unbinding will be seen in 10-25% of all force-distance cycles if the mica is densely covered with avidin [Riener et al., 2003]. (**C**) Specific block of avidin on mica with free biotin (0.1 mg/mL = 0.4 mM [Kamruzzahan et al., 2006; Ebner et al., 2007]).

Here, we describe an alternative testing method which uses a macroscopic enzyme assay. The procedure is illustrated in Figure 2. The AFM cantilever is mimicked by flat chips which consist of the same material as the AFM cantilevers (typically silicon nitride). The chips are cut into small squares (5 mm × 5 mm) and subjected to the same chemical steps as shown in Figure 1A for AFM tips:

1. aminofunctionalization
2. coupling of biotin-PEG-NHS

Consequently, biotin groups are tethered to the chip surface via long, flexible PEG chains (Figure 2A). The number of biotin groups (per μm^2) is then "counted" by measuring the amount of a labeled avidin molecule which binds to the biotin groups. Similar as in ELISA or Western blotting, the label is neither radioactive nor fluorescent. Instead, the label consists of an enzyme molecule (peroxidase) which is covalently connected to avidin.

➔ Text continues on page 5.

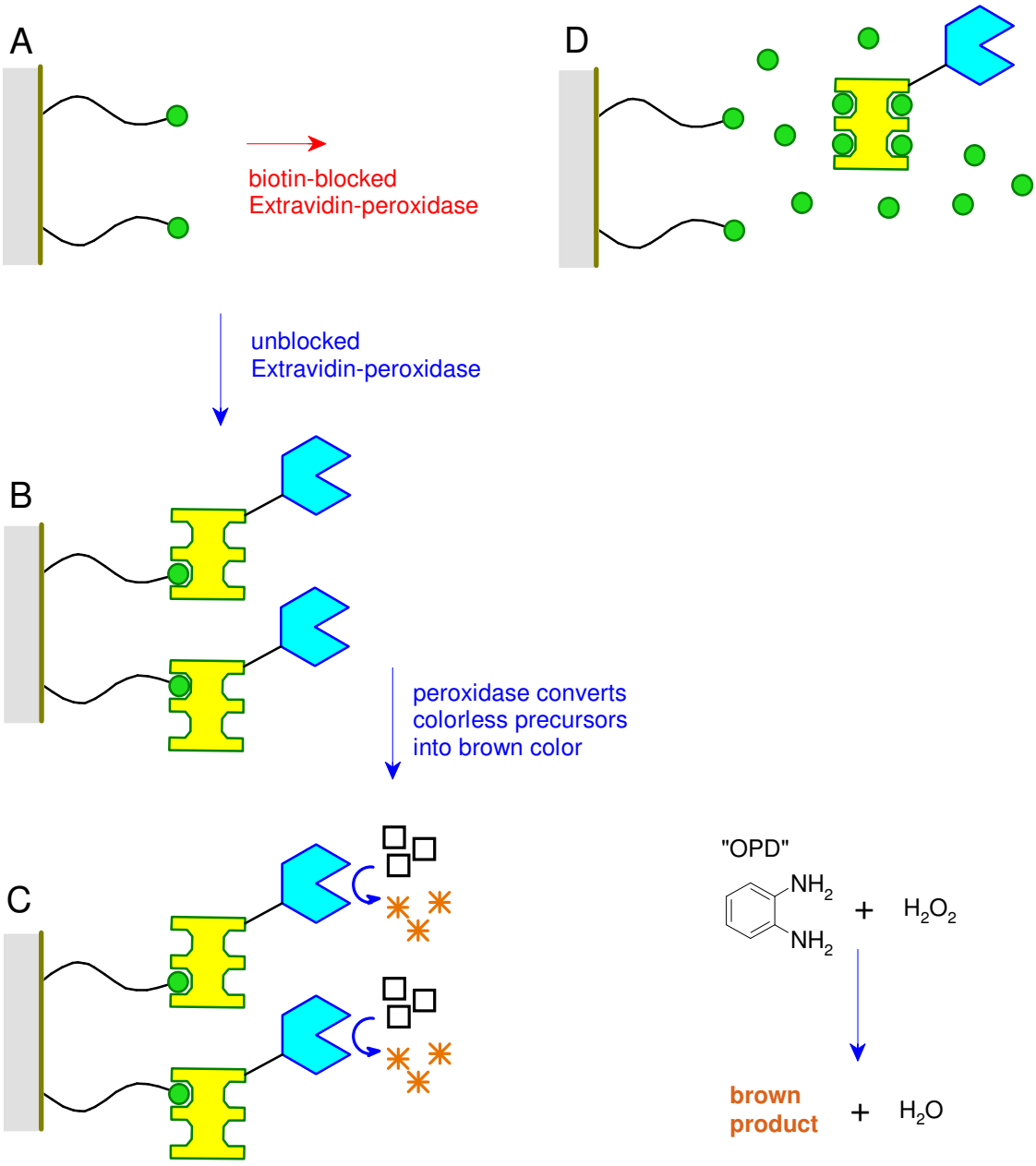

Figure 2: Enzymatic assay for the lateral density of biotin groups on surfaces. (**A**) A silicon nitride chip (5 mm × 5 mm) with PEG-linked biotin groups is prepared by the same procedure as shown for AFM cantilevers in Figure 1A. (**B**) Extravidin-peroxidase is bound to the biotin groups. Unbound Extravidin-peroxidase is washed away. (**C**) Addition of o-phenylene diamine and H_2O_2 provides for production of **brown color** by the peroxidase. (**D**) Control experiment with biotin-blocked Extravidin-peroxidase. This control shows the extent of nonspecific adsorption to the chip surface (compare Figure 3B).

←→ Continuation of text from page 3

The commercial name of the adduct between avidin and enzyme is "Extravidin-peroxidase". The term "Extravidin" denotes a modified version of avidin which shows much less nonspecific adsorption to surfaces than wild-type avidin. The peroxidase part is the same as used in ELISA or in Western blotting (also termed horse radish peroxidase, HRP).

The mechanism of the assay is illustrated in Figure 2. Incubation of the chip affords binding of Extravidin-peroxidase to the surface-linked biotin groups (**green spheres**). All loosely adhering Extravidin-peroxidase must be washed away. Only then, a mixture of o-phenylene diamine (OPD) and H_2O_2 (hydrogen peroxide) is added. Now the surface-bound peroxidase uses H_2O_2 to convert the colorless OPD into a soluble brown product.

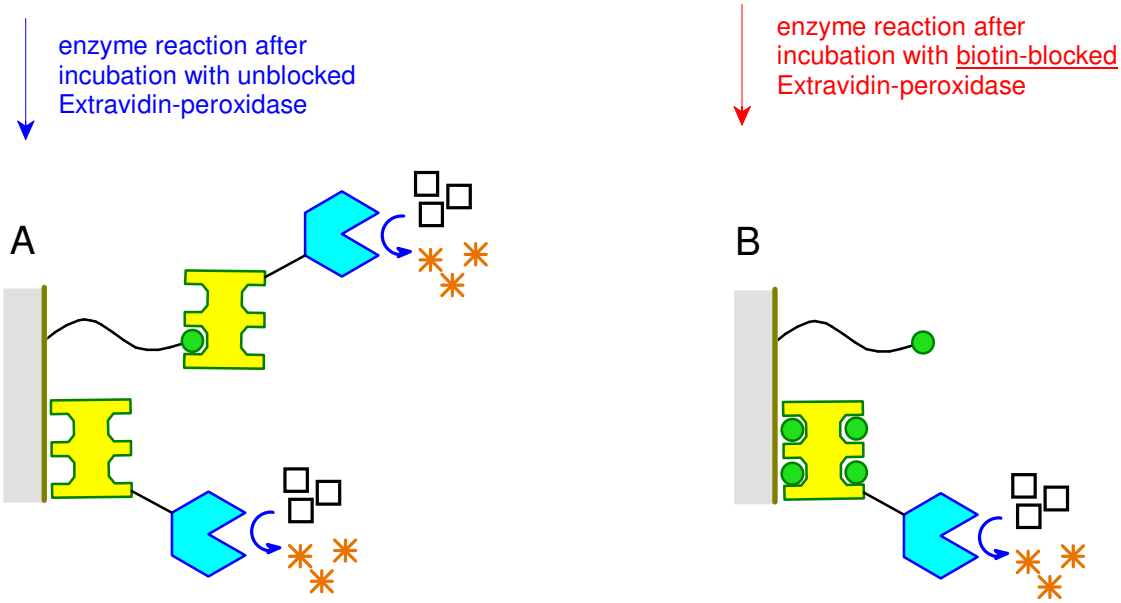

Figure 3: Discrimination between specific and nonspecific binding of Extravidin-peroxidase. (**A**) Extravidin-peroxidase is immobilized on the chip (i) by specific binding to surface-linked biotin and (ii) by nonspecific adsorption to the surface. (**B**) In presence of free biotin, all biotin-binding sites are blocked and Extravidin-peroxidase is immobilized by nonspecific adsorption only. The extent of nonspecific binding is minimized by including 0.5% Tween-20 in during incubation of the surface with Extravidin-peroxidase. Subtraction of nonspecific binding (panel B) from total binding (panel A) allows calculation of specific binding.

Figure 2D shows an important parallel control for explicit quantification of nonspecific binding. Extravidin-peroxidase is premixed with an excess of free biotin which prevents specific binding to the surface-linked biotin groups on the chip surface. Nevertheless,

Extravidin-peroxidase can still be immobilized on the chip surface by nonspecific adsorption (see Figure 3B).

In absence of free biotin (Figure 3A), two mechanisms contribute to the immobilization of Extravidin-peroxidase to the chip:

1. specific binding of Extravidin-peroxidase to surface-linked biotin groups
2. and nonspecific adsorption of Extravidin-peroxidase to the chip surface.

The number of specifically bound Extravidin-peroxidase molecules is obtained by subtracting the number of nonspecifically bound molecules (determined as shown in Figure 3B) from the total number bound molecules (determined as shown in Figure 3A).

It is important to include a suitable detergent (0.5% Tween-20) when incubating the chip with Extravidin-peroxidase. Tween-20 reduces nonspecific adsorption of Extravidin-peroxidase in the sample (Figure 3A) and in the control (Figure 3B) to a level which is much lower than specific binding via surface linked biotin groups (see Figure 3A).

The practical assay procedure is illustrated in Figure 4.

- For measurement of one chip (Figure 4A), the cuvette is filled with 3 mL assay buffer which contains the enzyme substrates OPD and hydrogen peroxide. The absorbance (**A_{490}**) of the cuvette is recorded. Then, the washed chip with the bound Extravidin-peroxidase is immersed for a defined time interval (e.g., 1 min) while stirring the solution. At the end of the time interval (**Δt**) the chip is removed and stored in a beaker with assay buffer. The absorbance (**A_{490}**) of the cuvette is recorded again. If the magnitude of the absorbance change (ΔA_{490}) is too small, then the chip can be re-immersed for a longer time interval (e.g., 5 min) after which A_{490} is recorded again.
- For measurement of another chip (same as Figure 4A), the cuvette is filled with fresh assay buffer (containing OPD and hydrogen peroxide) and the next chip is immersed for a defined time interval (**Δt**), whereupon the absorbance change (**ΔA_{490}**) is determined.
- For calibration of the enzyme activity, the cuvette is filled with fresh assay buffer and a defined amount of Extravidin-Peroxidase is added at t = 0 min. The absorbance (**A_{490}**) is monitored over time, and the slope (**$\Delta A_{490}/\Delta t$**) is calculated from the linear absorbance change.

We then compare the color increase ($\Delta A_{490}/\Delta t$) from the known number of soluble enzyme molecules (Extravidin-peroxidase) with the color increase $\Delta A_{490}/\Delta t$ from unknown number of the chip-bound enzyme molecules, using a simple linear relationship:

unknown enzyme number on the chip / *known enzyme number in solution*
= **$\Delta A_{490}/\Delta t$** *with chip-bound enzymes* / **$\Delta A_{490}/\Delta t$** *with soluble enzymes*

hermann.gruber@jku.at

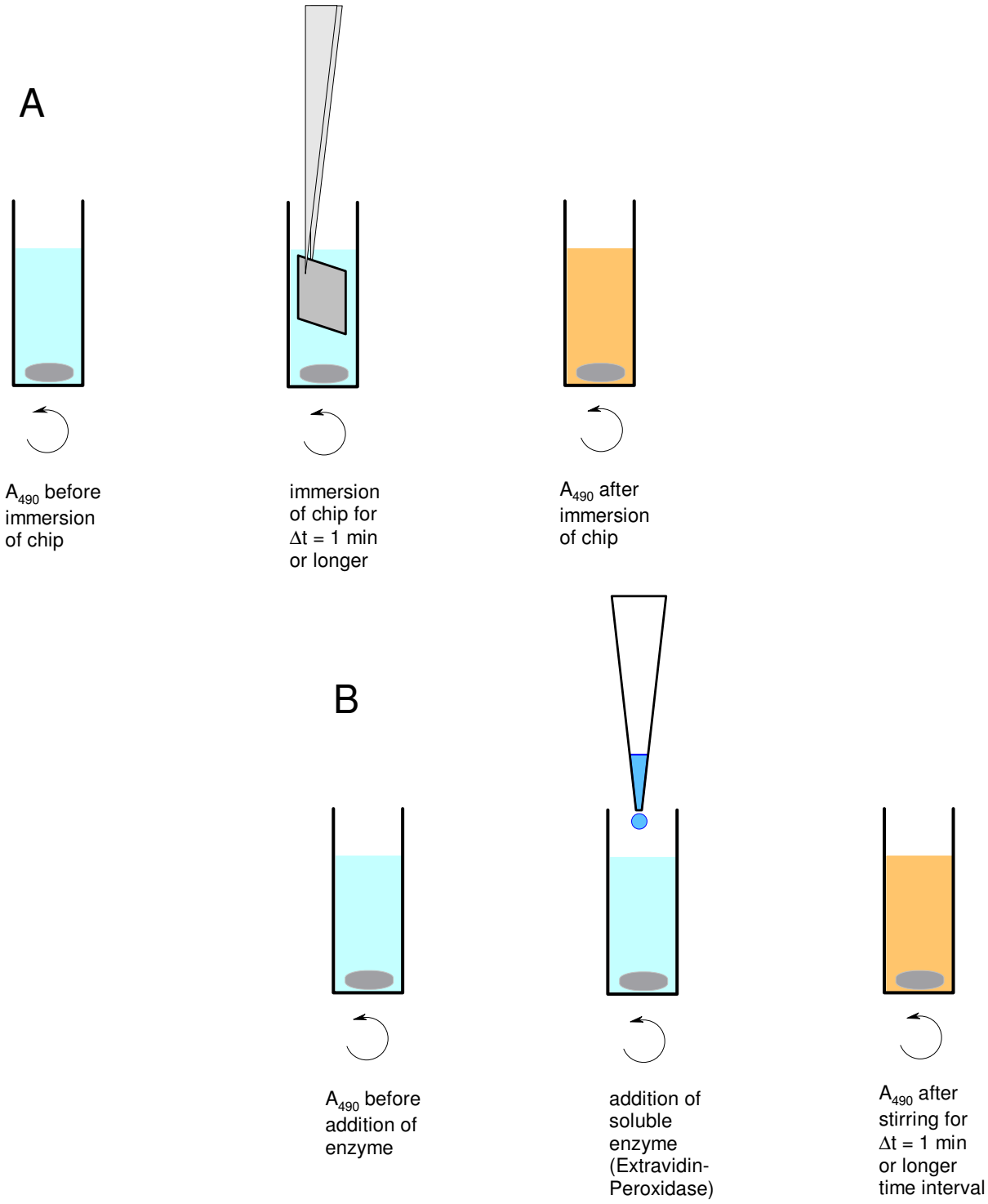

Figure 4: Assay procedure. (**A**) Immersion of chip causes color production in the solution by chip-bound Extravidin-peroxidase. (**B**) Parallel calibration procedure where a known number of Extravidin-peroxidase molecules is added to the solution, causing color production at the same rate per minute and per enzyme molecule. Please, note that stirring is indispensable. Stirring can also be done outside the spectrophotometer.

The number of enzyme molecules in solution (in the calibration run) is estimated as described in the following: The Extravidin-peroxidase stock solution from Sigma-Aldrich (product E2886) has an average protein concentration of 2 mg/mL and an average peroxidase/avidin ratio of 0.7. Consequently, the MW of Extravidin-peroxidase is assumed as 120 kDa and the molar concentration of Extravidin-peroxidase is assumed as 16.7 µM. The stock solution is diluted by a factor of 200 (yielding a concentration of 84 nM). 5 µL of this diluted sample is added to the cuvette (see Figure 4B). This corresponds to an amount of 5 µL × 84 nM = 420 fmol. Multiplication with Avogadro's number (6.023×10^{23} molecules in 1 mol) gives an absolute number of 2.5×10^{11} enzyme molecules in the cuvette.

> **The essential idea behind the assay is that each enzyme molecule (i.e., each Extravidin-peroxidase) produces the same amount of colored molecules in a fixed time interval (1 min), whether the enzyme is in free solution of sitting on the chip surface.**

This condition is implemented by adjusting sufficiently high concentrations of OPD and hydrogen peroxide, so that each enzyme molecule is driven at its maximal speed (whether it is in free solution or bound on the chip). Stirring is important to achieve continuous replacement of consumed OPD and hydrogen peroxide by fresh enzyme substrates. In spite of stirring, we have a thin unstirred layer of liquid next to the chip surface. Transport across the unstirred layer occurs via diffusion only, therefore the concentration of OPD and hydrogen peroxide next to the surface is lower than in bulk solution. In compensation for this effect, the concentrations of OPD and hydrogen peroxide in the bulk solution are further increased to a level which ensures saturation of all enzyme molecules, whether they are in free solution or sitting on the chip surface.

The take-home lesson from the previous paragraph is simple: Just stick to the specified assay conditions and don't forget to stir well! Please, note that stirring can be done either inside or outside the spectrometer. In the latter case, the cuvette is inserted into the spectrometer before and after stirring with an immersed chip. – If you don't have a magnetic stirrer then you could move the chip up and down as quickly as possible with the forceps. Be aware, however, that insufficient stirring can compromise the reproducibility of your data.

Procedure:

The above described experiments involve three major steps:

1. chip cleaning and aminofunctionalization
2. Coupling of biotin-PEG-NHS to the amino groups on the chip
3. Counting of the biotin groups on the chip surface

Cutting of the chips

A silicon nitride chip is weighed and its area is measured. The "specific weight per area" is calculated (in mg per mm^2). Then the chip is cut into squares (5 mm × 5 mm). If the chips are not perfectly regular squares then their exact area can be determined by weighing and divided by the "specific weight per area" to calculate the exact area. Such area determination, however, is only done at the end of the whole procedure.

For a representative data set, six chips should be derivatized. A group of three chips should be evaluated with unblocked EAP reagent and another group of three chips with blocked EAP reagent.

Cleaning and aminofunctionalization

The experimental details are described in the manual *AFM_tip_aminofunctionalization*. The only difference is that you apply the method to silicon nitride chips instead of AFM cantilevers. This does not cause any change of the procedure because aminofunctionalization is performed in large volumes anyway.

Coupling of Biotin-PEG-NHS

The experimental details are described in the manual *AFM_tip_with_biotin*, with minor adaptations:

Because of the larger size of the chips, a small glass beaker (5 mL or 10 mL) should be used as reaction chamber. Two (or three) 1 mg portions of Biotin-PEG-NHS are dissolved in 2 mL (or 3 mL) chloroform and 60 µL (or 90 µL) of triethylamine is added. The beaker is carefully covered with several layers of aluminum foil which is pressed onto the upper rim to minimize evaporation losses. All other details are the same as in the manual *AFM_tip_with_biotin*.

Counting of biotin groups on the chip surface:

- The bottom of a disposable Petri dish is covered with Parafilm (unstretched).
- The chips are washed in PBS (3 × 10 min) and water (3 × 1 min) and blown dry with a stream of nitrogen. (Be sure to omit water and to avoid drying if you have a biotinylated protein on the chip. In this case you simply remove most of the buffer by holding the chip in upright position with one edge on Kleenex tissue to blot off the liquid.)
- The chips are placed on the Parafilm in the Petri dish.
- 50 µL of EAP reagent (blocked or unblocked!) is placed on each chip. For representative data, the assay is performed in triplicates. This means that three chips are

covered with unblocked EAP reagent (50 µL per chip) and another group of three chips with blocked EAP reagent (50 µL per chip).

- The Petri dish is covered with its lid and the chips are incubated for 60 min at room temperature.
- The chips are washed in PBT (3 × 10 min) and in PBS (3 × 10 min).
- A UV-vis spectrophotometer is set to an absorption wavelength of 490 nm.
- Two large cuvettes (for 3-4 mL assay volumes) are inserted into the spectrophotometer.
- The reference cuvette is filled with 3 mL water.
- Each chip is analyzed as described in the following box.

- The sample cuvette is filled with 3 mL OPD reagent and 30 µL of 3% H_2O_2 is added and mixed into the OPD reagent.
- A small beaker with 5 mL assay buffer is placed next to the spectrophotometer.
- The absorbance A_{490} of the sample cuvette is measured.
- The sample cuvette is mixed with magnetic stirring, either inside the spectrophotometer (if available), or on a magnetic stirrer outside the spectrophotometer (see Figure 4).
- The first chip is submerged inside the stirred solution with the help of a forceps (tweezer) for exactly 1 min.
- The chip is removed and immersed in the beaker which contains 5 mL assay buffer. This beaker serves as "parking place" for the chip.
- The absorbance A_{490} of the sample cuvette is measured. If $A_{490} < 0.1$ then the chip should again be immersed for a longer time interval to obtain $A_{490} > 0.1$. The lengths of the time intervals are written down.
- At the end of the second reaction period, the chip is again transferred into the beaker with the 5 mL assay buffer and the absorbance A_{490} of the sample cuvette is measured.
- The chip can be re-immersed several times. All reaction periods must be summed up to determine the total time span which the chip has spent in the sample cuvette, providing for color production by the chip-bound enzymes.
- The contents of the sample cuvette is disposed, and the cuvette is washed with water (5×).
- Fresh OPD reagent (3 mL) is filled into the sample cuvette, 30 µL 3% H_2O_2 is added, and the next chip is analyzed (see top of this box).

- Now we can calculate the absorbance increase ($\Delta A_{490}/\Delta t$) which was caused by each individual chip. ΔA_{490} is the total absorbance increase from one or several reaction periods, and Δt is the sum of all time periods of one chip.
- For calibration purposes, the sample cuvette is filled with 3 mL OPD reagent.
- 30 µL 3% H_2O_2 is added.
- The absorbance A_{490} of the sample cuvette is measured.
- 10 µL of the EAP reagent (a 20-fold dilution of the commercial stock solution) is added to 90 µL assay buffer to obtain a 200-fold dilution of the commercial stock solution.
- 5 µL of this 200-fold dilution of EAP reagent is added to the sample cuvette with stirring. This starts the enzyme reaction which causes a linear increase of A_{490} in the sample cuvette.
- Because of the linear slope, it is not a problem if mixing is performed outside the spectrophotometer and the initial time course of A_{490} is not monitored.
- Preferably, the time course of A_{490} is recorded with the "time scan mode" of the spectrophotometer. Alternatively, the value for A_{490} is repeatedly determined after each 1 min interval.
- Now we can calculate the absorbance increase ($\Delta A_{490}/\Delta t$) which was caused by the soluble EAP molecules in the sample cuvette.
- We then compare the color increase ($\Delta A490/\Delta t$) from the known number of soluble enzyme molecules (Extravidin-peroxidase) with the color increase ($\Delta A490/\Delta t$) from unknown number of the chip-bound enzyme molecules, using a simple linear relationship:

unknown enzyme number on the chip / *known enzyme number in solution*
= $\Delta A_{490}/\Delta t$ *with chip-bound enzymes* / $\Delta A_{490}/\Delta t$ *with soluble enzymes*

- The "known enzyme number in solution" can be estimated as 2.5×10^{11} molecules (see page 9).
- The numbers of enzymes per chip should be divided by the number of square microns per chip in order to obtain lateral densities (molecules/µm^2) which are easy to compare with literature data. Efficient functionalization means that 1000 – 2000 EAP molecules are bound on one µm^2 [Riener et al. 2003; Kamruzzahan et al. 2006; Ebner et al. 2007]. Only one face of the chip is considered because only one side of the chip has been incubated with EAP reagent. The 5 mm × 5 mm chip has an area of 25 mm^2 which corresponds to 25×10^6 µm^2.
- Triplicates are grouped for calculation of average values and standard deviations.

- The average value for blocked chips is subtracted from the average values for unblocked chips in order to calculate the lateral density EAP molecules which were specifically bound by biotin-PEG on the chip surface.

Notes:

-  Ortho-Phenylene diamine dihydrochloride (OPD) is carcinogenic! Do not breathe the dust. Wear disposable gloves and a lab coat.

- Please, consults the Notes of the manual *AFM_tip_aminofunctionalization* which is used for aminofunctionalization of the silicon nitride chips.
- Please, consults the Notes of the manual *AFM_tip_with biotin* which is used for coupling of Biotin-PEG-NHS to the silicon nitride chips.
- With little adaptation, you can use this manual for examination of protein coupling via Aldehyde-PEG-NHS or Acetal-PEG-NHS. You simply replace Biotin-PEG-NHS in the above procedure with Aldehyde-PEG-NHS or Acetal-PEG-NHS. Subsequently, Acetal functions are cleaved with 1% citric acid (10-15 min at room temperature), as describe in the manual AFM_tip_with_acetal. Then, you couple some commercial biotinylated protein (e.g., Sigma-Aldrich P2165-2MG, Protein A-Biotin), exactly described in the manuals AFM_tip_with_aldehyde or AFM_tip_with_acetal. After protein coupling you proceed to "Counting of biotin groups on the chip surface" (pg 10). Be aware that biotinylated proteins are only stable in buffer (not in water) and that drying is also forbidden.
- Because of the low lateral densities of the PEG linkers, it is not possible to use standard surface analytics like XPS or similar techniques. We typically found 1000 – 2000 biotin groups which were linked to the chip surface vial PEG chains. For comparison, the theoretical maximum for a dense monolayer of alkyl chains is 3×10^6 molecules per μm^2. This number is estimated from the area per fatty acid which is found in a compressed monolayer (gel phase, 0.3 nm^2 surface area per molecule).
- Please, note that Extravidin-peroxidase is a statistical adduct of modified avidin and peroxidase. It may contain several copies of each protein. Our estimate of 120 kDa for one adduct is a simplification [Riener et al. 2003; Kamruzzahan et al. 2006]. Other estimates for the MW of Extravidin-peroxidase would multiply the biotin group density values by a constant factor. We suggest sticking to the old estimate of 120 kDa because this helps to compare your data with our literature data.

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 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**).

PBS with Tween (**PBST**) is prepared by mixing Tween-20 with PBS at a final Tween concentration of 0.5% (w/v). Tween-20 is a viscous liquid and it is best pipetted with the blue tip of a digital pipette after having cut away few millimiters from the tip to widen the tip outlet. Slightly less than 1 mL of Tween-20 is pipetted into a pre-weighed measuring cylinder and the weight of the Tween quantity is determined. The weight (in grams) is multiplied by 200 to calculate the final volume of the solution (in milliliters). PBS is filled into the measuring cylinder to obtain this final volume. The cylinder is closed with Parafilm and the solution is carefully mixed. For example, if the weight of Tween in the cylinder is 917 mg then the desired final volume is 0.917 g × 200 = 183.4 mL.

Assay buffer: 50 mM citric acid (192.13 g/mol), adjusted to pH 5.50 with NaOH. Dissolve 2.4 g citric acid in water (~220 ml), adjust the pH to 5.50 with NaOH solution. You can use 20% NaOH at the beginning and 2% NaOH when coming close to pH 5.50.

Biotin stock solution (**10 mM**) is prepared by dissolving and mixing the components listed below in about 40 mL water, transferring the solution into a 50 mL volumetric flask (or a measuring cylinder), and adding water to give exactly 50 mL volume. Mix carefully. The pH will automatically be >7 which is required to dissolve biotin and EDTA completely. No pH adjustment is required. Aliquots can be stored at -20°C for up to several years.

- 5 mmol Na_2HPO_4 (in case of anhydrous dibasic sodium phosphate: 141.96 g/mol × 0.005 mol = **0.710 g anhydrous Na_2HPO_4**; in case of the heptahydrate: 268.07 g/mol × 0.005 mol = **1.340 g $Na_2HPO_4.7H_2O$**)
- 0.05 mmol EDTA disodium salt (372.24 g/mol × 0.00005 mol = **0.018 g EDTA disodium salt**).
- 0.5 mmol biotin (244.3 g/mol × 0.0005 mol = **0.122 g biotin powder**)

At room temperature, biotin rapidly degrades by oxidation [*Ebner et al., 2008 Methods in Molecular Biology. Volume 418: Avidin-Biotin Interactions. Methods and Applications (McMahon, R. J., Ed.) pp 73-88, Chapter 7, Humana Press, Totowa, N.J.*]. Exposure to room temperature should be kept to a minimum!

Unblocked EAP reagent is **freshly** prepared by diluting the stock solution from Sigma-Aldrich (product E2886, containing ~ 2 mg/mL Extravidin-peroxidase) with 19 volumes of PBT. For example, 50 µL Extravidin-peroxidase stock solution is mixed with 950 µL PBT and kept on ice.

Blocked EAP reagent is **freshly** prepared from unblocked EAP reagent by adding 0.02 volumes of 10 mM biotin stock solution. For example, 500 µL unblocked EAP reagent is mixed with 10 µL 10 mM biotin stock solution and kept on ice.

OPD reagent is prepared by dissolving one 10 mg tablet of o-phenylene dihydrochloride (**OPD**, Sigma P8287-50TAB) in assay buffer at a final concentration of 0.8 mg/mL. For this purpose, one tablet OPD (10 mg) is dissolved in 12.5 mL of assay buffer.

3% hydrogen peroxide (H_2O_2) can be prepared by one of two ways.

In the typical chemistry lab you will find concentrated H_2O_2 which has a concentration of 30%. This stock solution must be diluted with 9 volumes water to obtain 3% H_2O_2. For example, 100 µL of 30% H_2O_2 is mixed with 900 µL water to obtain 1 mL of 3% H_2O_2.

In the drug store (or pharmacy) you can purchase 3% H_2O_2 which can directly be used as reagent in this assay.

Literature on estimation of biotin group density with Extravidin-peroxidase

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Risk and Safety

	**Chloroform** ($CHCl_3$): toxic, co-carcinogenic, H302-H315-H351-H373, P281, R22-38-40-48/20/22; S36/37
	**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
	**o-phenylene diamine dihydrochloride** (1,2-diaminobenzene dihydrochloride): toxic, carcinogenic, H302-H317-H319-H332-H341-H351-H410, P273-P280-P305 + P351 + P338-P501, R20/21-25-36-40-43-50/53-68 (Europe), S26-36/37-45-61 (Europe)
	**Hydrogen peroxide** (H_2O_2) 30%: strong oxidant, corrosive, H302-H318, P280-P305 + P351 + P338, R22-41 (Europe), S26-39 (Europe)

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.