

# Toward Multiprotein Nanoarrays Using Nanografting and DNA Directed Immobilization of Proteins

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## ABSTRACT

Atomic force microscopy nanografting was utilized to prepare DNA nanopatches of different sizes ( $200 \times 200$  to  $1000 \times 1000$  nm<sup>2</sup>) onto which DNA–protein conjugates can be anchored through DNA-directed immobilization. Height measurements were used to assess the binding of the proteins as well as their subsequent interaction with other components, such as antibodies. The results indicate that nanografted patch arrays are well suited for application in biosensing and could enable the fabrication of multifeature protein nanoarrays.

The development of protein arrays with feature sizes at the micrometer length scale is currently of great interest for biomedical diagnostics and life sciences because these devices promise to evolve into a powerful technological platform for high-throughput analysis of biomolecular interactions with low requirements on the sample amount and hands-on processing time.<sup>1–4</sup> An important area in the developments of protein and small molecule arrays concerns their further miniaturization to the nanometer regime. Although various methods have been developed based on, e.g., dip-pen nanolithography,<sup>5–7</sup> SNOM lithography,<sup>8</sup> or micro contact printing,<sup>9</sup> it is currently still very difficult to fabricate nanoarrays containing multiple features, such as a range of different proteins or small-molecule ligands. We here report a novel approach for the fabrication of protein nanoarrays, which takes advantage of atomic force microscopy (AFM)-based nanografting, previously developed and applied for hybridization studies of DNA and other nucleic acids,<sup>10–14</sup> and DNA-directed immobilization (DDI) of semi-synthetic protein–DNA conjugates. The latter method, which takes advantage of the specific Watson–Crick hybridization

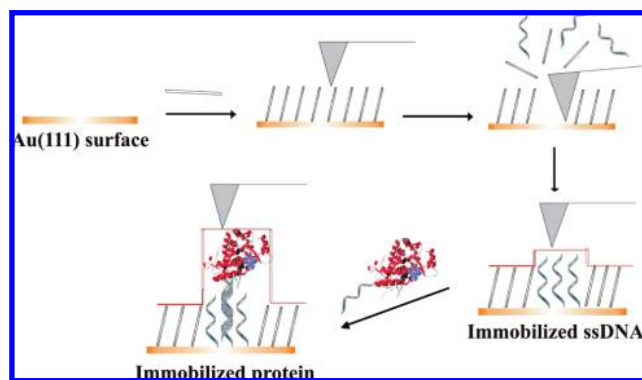


**Figure 1.** Schematic representation of the nanografting of ssDNA patches into a monolayer of ethylene glycol-modified thiols (top) and their subsequent functionalization with DNA–protein conjugates (bottom).

of oligonucleotide-modified proteins to surface-bound complementary oligomers, has previously been developed<sup>15</sup> and largely applied for the generation of self-assembled protein arrays at the micrometer length scale.<sup>16</sup> Although both AFM-based nanografting<sup>17</sup> and DDI<sup>18</sup> are by now considered established methods, it has not yet been shown that these two methods can indeed be combined and used to synergistically enable fabrication of protein nanoarrays with high control over lateral dimensions as well as options for reliable read-out, based on topographic AFM measurements. As depicted in Figure 1, DDI is used here to decorate nanometer-sized patches of single-stranded DNA (ssDNA), previously

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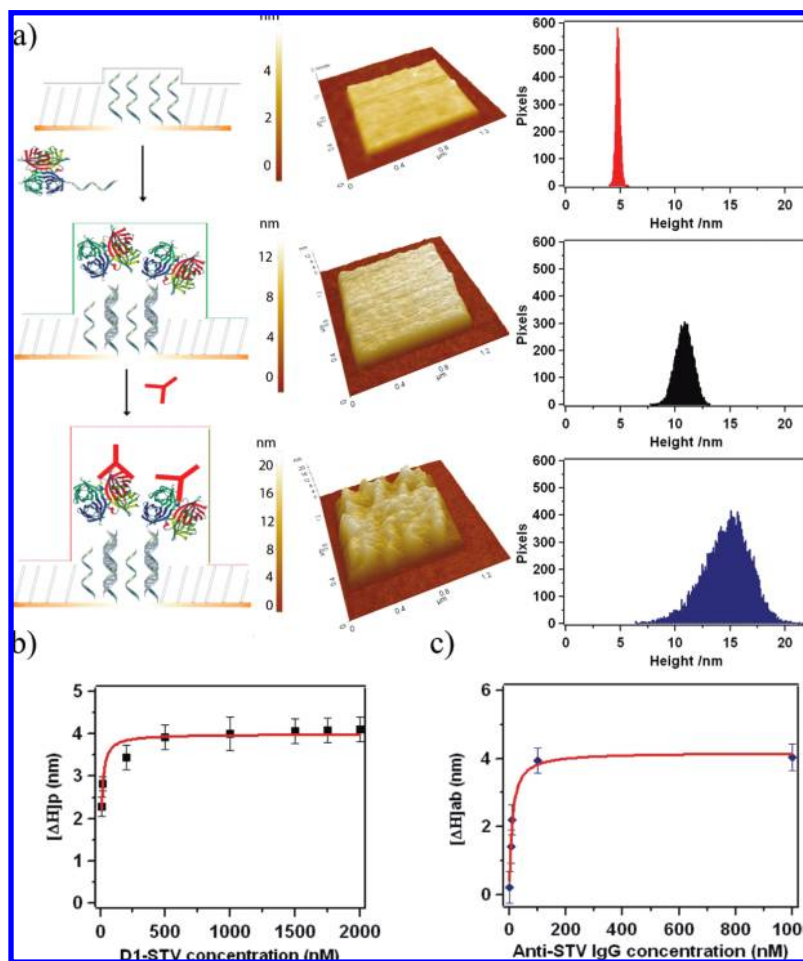


**Figure 2.** Nanografting-based preparation of protein arrays. (a) Schematic representation and resulting height histograms obtained for a single-patch DNA-directed sensor, generated from a covalent DNA–streptavidin conjugate (**D1-STV**), which was then used for the binding of the corresponding **anti-STV IgG** antibody. The curves in (b) and (c) show the changes in height as a consequence of variable protein concentrations. Note that the concentration of **D1-STV** used in all the experiments of antibody (**anti-STV IgG**) immobilization on STV modified patches was 1  $\mu\text{M}$ .

prepared by AFM-based nanografting of alkylthiol-modified gold surfaces,<sup>14</sup> with cDNA–protein conjugates. The integrity and biochemical functionality of the resulting protein arrays are demonstrated by AFM topography measurements and immunological assays, respectively.

To demonstrate the feasibility of our fabrication method (Figure 1), we initially generated nanografted patches of ssDNA within a monolayer of protein-repellent ethylene glycol-terminated alkylthiols ( $\text{HS}-(\text{CH}_2)_{11}-(\text{OCH}_2\text{CH}_2)_3-\text{OH}$ ) on a flat gold (111) substrate, using the previously published method.<sup>10,13,14</sup> Subsequently, proteins covalently modified with cDNA sequences were immobilized onto the nanopatch by means of DDI, thus producing a ready-to-use detector for protein interaction studies.

As shown in Figure 2, the substrate flatness allowed us to monitor both nanografting and subsequent protein decoration steps by means of AFM topographic height measurements. In particular, a  $1\ \mu\text{m} \times 1\ \mu\text{m}$  patch of alkylthiol-modified ssDNA **cD1** (for oligonucleotide sequences, see Supporting Information) was grafted within the monolayer of ethylene glycol alkylthiols. Subsequently, a covalent conjugate of streptavidin tethered with oligomer **D1** (**D1-STV**) was allowed to bind to the patch by sequence-specific DNA

hybridization. The gold surface was carefully washed with phosphate buffer (PBS, phosphate buffered saline) to remove any physically adsorbed molecules and imaged by AFM; all experiments were performed at least in triplicate and the given values and errors correspond to the mean values and standard deviation of height obtained from at least three independent patches. The resulting height profile of the initial ssDNA patch (average height  $\approx 6.5 \pm 0.5\ \text{nm}$ ) and the STV-modified patch ( $\approx 10.5 \pm 0.5\ \text{nm}$ ) indicated an increase in height of  $4 \pm 0.5\ \text{nm}$ , which is in good agreement with the size of native streptavidin (approximately  $4.2 \times 4.2 \times 5.6\ \text{nm}^3$ ), as determined by X-ray crystallography.<sup>19</sup> To independently identify STV and to demonstrate that the protein patches are suitable for protein–protein interaction studies, an anti-STV immunoglobulin G (**anti-STV IgG**) was allowed to bind to the surface. As shown in Figure 2a, the successful binding of IgG was clearly indicated by an increase in height from about 10.8 nm (STV patch histogram) to about 14.9 nm of the IgG-containing patch. It is known that IgG molecules have typical dimensions of  $14.5 \times 8.5 \times 4.0\ \text{nm}^3$ ,<sup>20</sup> and thus, the increase of about 4 nm suggested either that incomplete coverage of the patch occurred or that

**Table 1.** Summary of Binding Parameters Obtained from the Increase of the Height Observed on AFM Nanografted Patches upon Hybridization of DNA–STV Conjugate and Subsequent Binding of anti-STV IgG to This Patch

|                     | $K_D$ (nM)    | $h$ (nm)      |
|---------------------|---------------|---------------|
| <b>D1–STV</b>       | $8.1 \pm 1.3$ | $4.0 \pm 0.1$ |
| <b>anti-STV IgG</b> | $9.5 \pm 1.1$ | $4.2 \pm 0.1$ |

IgG molecules are immobilized to the patch in a horizontally oriented layer.

To further investigate the molecular interactions occurring on the nanografted DNA patch and to demonstrate that our method is well suited to the design of devices useful for biosensing, we determined quantitative affinity constants by using the differential height values, that is the height difference between the relevant and a reference patch (for definition see Figure S1 in Supporting Information). To this end, differential height values were plotted as a function of the target molecule concentration and data were fitted to the Langmuir equation with  $h$  and  $K_D$  as free parameters (for details, see Supporting Information) to yield the respective

dissociation constants  $K_D$ . The resulting plots and  $K_D$  values, obtained for both the binding of the ssDNA–STV conjugate (**D1–STV**) and the **anti-STV IgG** are shown in Figure 2b,c and Table 1, respectively. The  $K_D$  value obtained for the hybridization of the ssDNA–STV conjugate ( $8.1 \pm 1.3$  nM) is in good agreement with data obtained from BIAcore studies of similar DNA–STV conjugates (approximately 11–35 nM, depending on the oligonucleotide sequence).<sup>21</sup> Moreover, the  $K_D$  value obtained for the binding of **anti-STV IgG** ( $9.5 \pm 1.1$  nM) is consistent with data reported in the literature for this interaction.<sup>22</sup>

To further prove intactness and functionality of patch-immobilized STV molecules, we also investigated whether the STV can still bind biotinylated molecules. As shown in Figure S2 in Supporting Information, the patches decorated with **D1–STV** specifically bound biotinylated goat anti-mouse IgG, thus leading to an increase in patch height of  $4.8 \pm 0.5$  nm. This result therefore not only demonstrates preserved functionality of patch-immobilized STV but, being in full agreement with height values mentioned previously

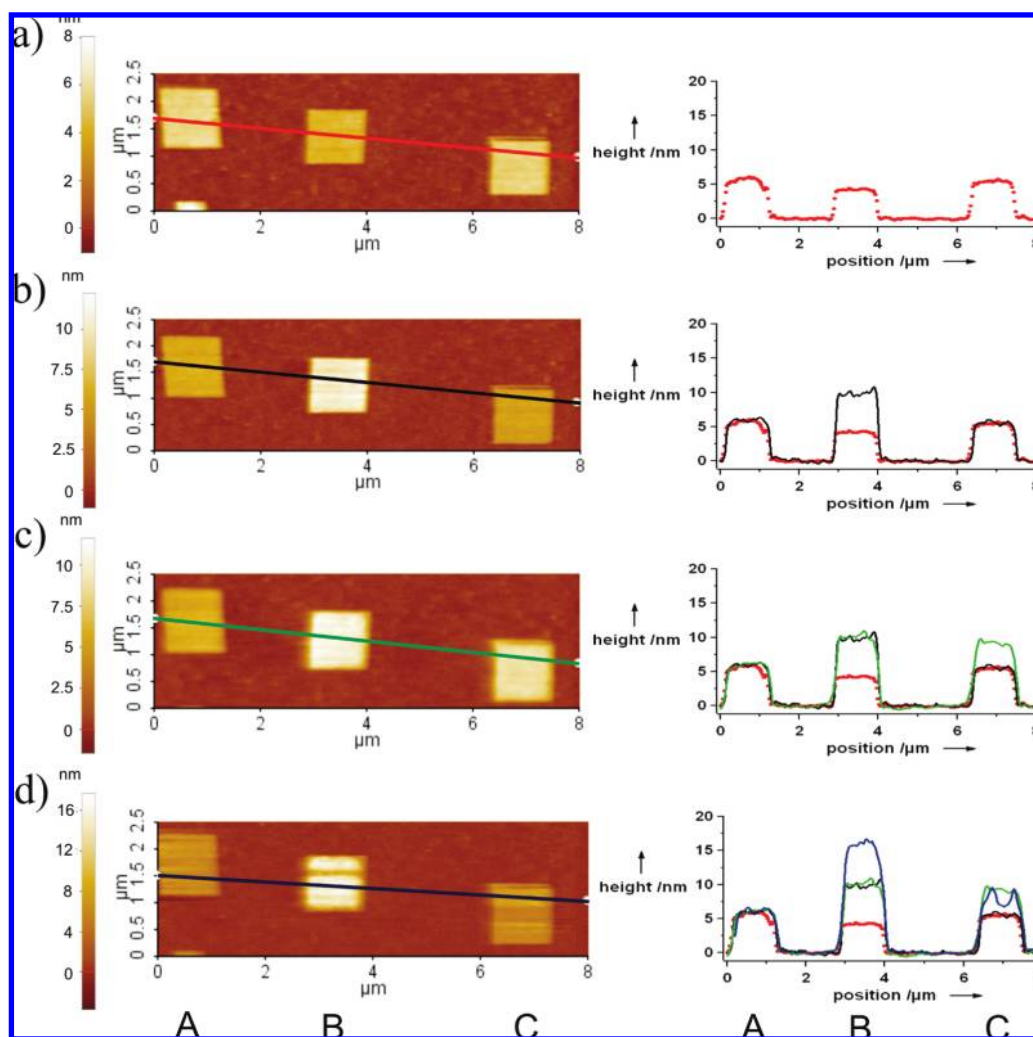


**Figure 3.** AFM topographic images ( $8 \times 2.5 \mu\text{m}^2$ ) and corresponding height profiles of a three-patch device, fabricated from DNA oligonucleotides **cd0** (patch A, left), **cd1** (patch B, middle), and **cd2** (patch C, right): (a) pattern after nanografting of ssDNA (red line); (b) pattern after hybridization of **D1–STV** conjugate (black line); (c) pattern obtained after further hybridization of **D2–HRP** conjugate (green line); (d) pattern obtained after further binding of **anti-STV IgG** (blue line in height profile).

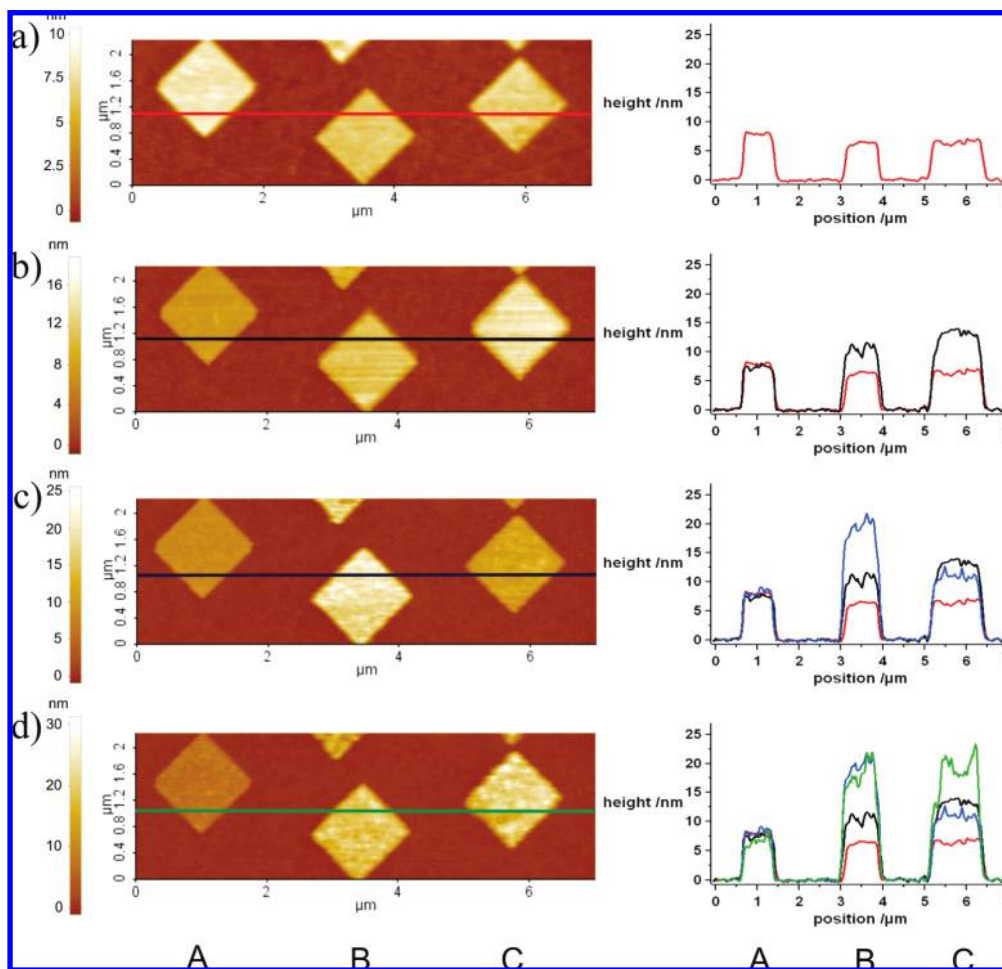


**Figure 4.** AFM topographic images ( $7 \times 2.2 \mu\text{m}^2$ ) and corresponding height profiles of antibody interaction with immobilized proteins. (a) Fabrication of ssDNA nanopatches, **cd0** (control patch A), **cd2** (patch B), and **cd1** (patch C), red line profile. (b) Patches after incubation with a solution containing **D2-STV** and **D1-GOx**. Note that the increase in height indicates successful immobilization of both conjugates, **D2-STV** (B) and **D1-GOx** (C), black line profile. (c) Patches after incubation with **anti-STV IgG**, dissolved in standardized human serum (BISEKO), blue line profile. (d) Patches after incubation with the **anti-GOx IgG** in BISEKO, green line profile.

for binding of the antibody directed against STV, also validates both our result and the method of detection used here, i.e., relative height measurements on a very flat surface.

To verify scalability to the nanometer regime of the combined nanografting/DDI approach, we also carried out a set of experiments, in which the size of the ssDNA patch was varied from  $200 \times 200 \text{ nm}^2$  to  $1000 \times 1000 \text{ nm}^2$  (Figure S3 in Supporting Information). We observed a consistent increase in patch height of about  $4 \pm 0.5 \text{ nm}$  upon hybridization of the ssDNA patches with the **D1-STV** conjugate, regardless of patch dimensions. In contrast, control experiments with STV lacking the covalently tethered oligomer revealed no increase in patch heights.

To further illustrate the applicability of our method to fabricate multiple-feature protein nanoarrays, a three patch device was generated (Figure 3), using three different DNA oligonucleotides (**cd0**, **cd1**, and **cd2**, sequences are listed in the Supporting Information). Oligomers **cd1** and **cd2** are complementary to conjugate **D1-STV** and a covalent DNA conjugate of horseradish peroxidase (**D2-HRP**), respectively, while **cd0** is a noncomplementary control sequence. After ssDNA patches A, B, and C were grafted (Figure 3a)

and washed with PBS buffer to remove physically adsorbed molecules, the gold substrate was immersed in a solution of **D1-STV** conjugate (1000 nM, PBS, pH 7.3). As shown in Figure 3b, AFM measurements revealed an increase in height ( $4.5 \pm 0.5 \text{ nm}$ ) only at patch B, which bears the complementary capture oligonucleotide. The same sample was then immersed in a solution containing the **D2-HRP** conjugate (500 nM, PBS, pH 7.3). Again, AFM imaging revealed an increase in height of  $3.8 \pm 0.5 \text{ nm}$  (Figure 3c) only for patch C, thereby clearly indicating specific surface immobilization of the **D2-HRP** conjugate. The dimension of HRP molecules is known from X-ray crystallography ( $4.0 \times 6.7 \times 11.7 \text{ nm}^3$ ),<sup>23</sup> and the observed increase in height of patch C is in good agreement with previous studies on HRP immobilization.<sup>24</sup>

The patch array was then probed with a solution of **anti-STV IgG** (1000 nM, PBS, pH 7.3). As expected, only the height of patch B, which contained **D1-STV** conjugate was increased (Figure 3d). In contrast, no significant changes were observed for patches A and C, and also, the background heights remained entirely unchanged in the course of this multistep experiment (Figure 3).

Similar results were obtained in a set of experiments concerning the simultaneous DNA-directed immobilization of DNA–HRP and DNA–GOx (GOx = glucose oxidase) conjugates on nanografted patches and the subsequent probing with an HRP specific antibody (Figure S4 in Supporting Information). A patch array was prepared by nanografting patches ( $1 \times 1 \mu\text{m}^2$ ) of **cd0** (control patch A), **cd2** (patch B), and **cd1** (patch C). After grafting, solutions containing **D2–HRP** or **D1–GOx** were allowed to bind to the patch array by means of DDI. From the increase in height it was clearly evident that the immobilization occurred in a sequence specific manner and no cross reactivity was apparent. Subsequent antibody binding experiments showed the presence of HRP on patch B (**cd2**), while the control patch showed no increase in height both after the hybridization of protein conjugates and antibody binding. This result clearly indicated specificity of DNA directed immobilization of HRP conjugate as well as the anti-HRP antibody–antigen interaction.

Given the successful demonstration of multiplex immunoassays on the nanografted patches, we then challenged our system with the biomedically relevant matrix of human serum. In particular, we investigated whether the nanografting/topography platform is suitable to detect a target protein in the complex matrix of standardized human serum (BISEKO). To this end, a patch array was grafted, containing **cd0** (control patch A), **cd2** (patch B), and **cd1** (patch C), and **D2–STV** and **D1–GOx** were allowed to bind to this array (Figure 4). Two specific antibodies, **anti-STV IgG** and **anti-GOx IgG** dissolved in BISEKO matrix, were allowed to bind to this array, and each binding was monitored sequentially by topography measurements. It is clear from the results shown in parts c and d of Figure 4, respectively, that both antibodies could be detected and no indication of nonspecific binding of either matrix components or target proteins disturbed these analyses. The high specificity of both DDI and IgG binding was also confirmed for our samples by standard microarray analyses (Figure S5 in Supporting Information), which also clearly indicated the absence of any nonspecific binding and cross-reactivity.

In conclusion, we reported here on the use of nanografted oligonucleotide patches for specific immobilization of proteins and on their subsequent use for label-free detection of protein binding interactions. In particular, the observed specificity of biomolecular recognition and the lack of nonspecific binding clearly illustrate that nanografted patch arrays are well suited for applications in biosensing. Since this platform can be conveniently scaled down to the nanometer regime, it should prove useful for the fabrication of multifeature protein nanoarrays, useful for multiplexed analysis of biomolecular recognition events and other applications in nanobiotechnology, such as single-cell proteomics.

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**Supporting Information Available:** Experimental procedures, details on the nanopatch stability, and STV–biotinylated antibody binding. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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# **Towards Multiprotein Nanochips Using Nanografting and DNA Directed Immobilization of Proteins**

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## **Supporting Information**

## Materials and Methods

**Chemicals:** Thiol- and amino -modified oligonucleotides,

**cD0** (SH or NH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-5'-TTCGGCTCATACTCTGACTGTA-3'),

**cD1** (SH or NH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-5'CTTCACGATTGCCACTTTCCAC-3'),

**cD2** (SH or NH<sub>2</sub>-(CH<sub>2</sub>)<sub>6</sub>-5'-CTTATCGCTTTATGACCGGACC-3') and

non-modified oligonucleotide, **D0** (5'-TACAGTCAGAGTATGAGCCGAA-3')

were all purchased as HPLC purified grade from Biomers GmbH (Ulm, Germany). The final concentration of all oligomers was 100μM. Sodium chloride (NaCl), ethylenediaminetetraacetic acid (EDTA) and phosphate buffered saline (PBS) tablets were purchased from Sigma. The buffers, TE (1M NaCl, 10mM Tris, 1mM EDTA, pH 6.9) and PBS (10mM phosphate buffer, 2.7mM potassium chloride (KCl) and 137mM NaCl, pH 7.3) were prepared using MilliQ water (resistance > 18 MΩcm) and filtered before use. Sulfo-succinimidyl-4-(N-maleimido-methyl)-cyclohexan-1-carboxylate (sSMCC) was obtained from Pierce and dithiothreitol (DTT) from Fluka.

**Proteins:** Horseradish peroxidase (HRP) and glucose oxidase (GOx) were purchased from Sigma. Streptavidin (STV) was expressed as recombinant core streptavidin in *E. coli* using a published protocol and purification procedures<sup>1</sup> and all of the used antibodies were obtained from Abcam (Cambridge, UK).

**AFM:** All AFM experiments were carried out using an XE-100 PARK AFM system, equipped with a custom liquid cell. All Nanografting (NG) experiments were performed by contact-mode AFM with standard ("hard") silicon cantilevers (MikroMasch, nominal spring constants 0.63Nm<sup>-1</sup>, tip radius < 10nm) at an applied force of 80-100nN and a tip speed of 500nm/sec, in the presence of thiolated single stranded DNA (ssDNA) solution (10 μM, dissolved in a 1:1 mixture of TE buffer and ethanol). Topographic images of NG-generated ssDNA nano-patches were recorded with standard ("soft") silicon cantilevers (MikroMasch, nominal spring constants 0.03Nm<sup>-1</sup>, tip radius < 10nm) in modified PBS buffer (1M PBS, 1mM EDTA, pH 7.2). The substrates were washed thoroughly with TE and PBS buffers before and after the hybridization, respectively, to remove physically adsorbed molecules.

**Monolayer preparation:** For preparing a protein resistant monolayer on ultra flat (111) gold substrate, a 100nm thick gold film was deposited on freshly cleaved mica sheets (Mica New York Corp., clear ruby muscovite) at a pressure of about  $10^{-5}$  mbar in an electron-beam evaporator at a rate of 0.1nm/sec. In the present work, Epoxy SU8-100 (negative tone photoresist, MicroChem) was used as a solid support. A drop of SU-8100 was placed over small slides (few millimeters in size) of gold substrate and then cured to form SU8-100/gold/mica sandwich. The gold-mica interface was uncovered mechanically, and immediately immersed into a freshly prepared 100 $\mu$ M solution of top oligo-ethylene-glycol3 terminated alkylthiols (TOEG3, SH-(CH<sub>2</sub>)<sub>11</sub>-(O-CH<sub>2</sub>-CH<sub>2</sub>)<sub>3</sub>-OH, Prochimia) in pure ethanol (Fluka, purity  $\geq$  99.8 %), and incubated for 15 hours at room temperature. The resulting SAM was then rinsed with ethanol and dried under a mild stream of nitrogen. AFM was used to measure the roughness of the SAM, which was typically in the range of a few angstroms.

**DNA-protein conjugates preparation:** The **D1-STV**, **D2-STV**, **D2-HRP** and **D1-GOx** conjugates were prepared using the bifunctional crosslinker sSMCC (sulfo-succinimidyl-4-(N-maleimido-methyl)-cyclohexan-1-carboxylate), following a previously described method.<sup>2,3</sup> Briefly, 100  $\mu$ L of a 100  $\mu$ M solution of the corresponding 5'-thiol modified oligonucleotide in TE buffer was mixed with 60  $\mu$ L DTT (1M) and incubated overnight at 37°C to reduce any disulfide bonds formed upon storage of the oligonucleotide. 200  $\mu$ L of 200  $\mu$ M protein solution (in phosphate buffer, pH 7.2) were incubated for 1 h at 37°C with sSMCC (2 mg in 60  $\mu$ L DMF). Both DNA and protein solution were purified by two consecutive gel-filtration chromatography steps using NAP5 and NAP10 size exclusion columns (Pharmacia). The purified DNA and protein solutions, each of which had a volume of 1.5 ml, were then combined and incubated in the dark at room temperature for 3 h. The reaction mixture was concentrated to approx 300  $\mu$ L by ultrafiltration (Centricon 30, Milipore) and the buffer was exchanged to Tris (20 mM, pH 8.3) during this step. The conjugates were purified by anion exchange chromatography on a MonoQ HR 5/5 column (Pharmacia) using linear gradient over 25 min (AKTA purifier, Amersham Bioscience, buffer A: 20 mM Tris A pH 8.3 and buffer B: 20mM Tris A and 1.5 M NaCl). The concentration was determined spectrophotometrically, as described previously.<sup>2,3</sup>

**Protein immobilization:** Nanografting was carried out as described earlier.<sup>4</sup> Briefly, the AFM tip scans the selected area at relatively large forces in the presence of thiolated ssDNA molecules in solution. Under the tip load, molecules from the liquid solution phase replace, in a localized manner, molecules removed from the TOEG3 SAM and self-assemble on the gold surface. In our previous studies, we have demonstrated that the density of DNA confined by NG within a nanosized area on the surface is proportional to the number of sweeps driven by the AFM tip in a particular area.<sup>5,6</sup> For quantitative studies, we have introduced a grafting parameter that is the ratio, namely  $S/A$ , between the total area ( $S$ ) that is covered by the tip during nanografting and the area of the patch ( $A$ ). Basically  $S/A$  can be expressed as the density of nanografted scanning lines multiplied by the tip size at the point of contact with the surface. Therefore, the higher the density of strokes applied by the tip, the higher is  $S/A$ . For instance, for  $S/A = 1$ , the nanografted scanning lines do barely touch each other, while for  $S/A = 5$ , the distance between adjacent scanning lines is  $1/5$  of the tip size, and in this way each area in the nano-patch has been “overwritten” five times. Nanopatches of ssDNA molecules (e.g., cD1) of various  $S/A$  were fabricated. To immobilize the protein, the sample was then incubated in a solution containing the DNA-protein conjugate (e.g., **D1-STV**, 2  $\mu$ M in PBS buffer) for 2h at room temperature.

**Antibody binding:** To accomplish the binding of antibodies, the substrate containing immobilized protein features, such as **D1-STV** and **D2-HRP**, were incubated with a solution of polyclonal antibodies (rabbit **anti-STV IgG**, concentrations ranging from 500pM to 1000nM, and/or rat **anti-HRP IgG** (10nM to 1000nM) in PBS buffer for 3h at RT. For the set of serum experiments, the binding of antibodies were carried out by incubating the immobilized protein patterns (**D2-STV** and **D1-GOx**) with a solution of the mixture of 50% standardized human serum (BISEKO) and **anti-STV IgG** (1000nM, PBS, pH 7.4) for 30 min. After washing, the substrate was then incubated with a solution of **anti-GOx IgG** (1000nM, PBS, pH 7.4) dissolved in 50% serum for 30 min.

Subsequent to incubation, the substrates were thoroughly washed with PBS buffer to remove any non-specifically adsorbed materials prior to AFM measurements.

**Determination of dissociation constants.** To determine the dissociation constants for the binding of the protein conjugate **D1-STV** and the **anti-STV IgG** within the nanografted DNA patches, differential height values (Fig S1), that is the height difference between the relevant patch and a reference patch (example in Fig S1), were measured as a function of probe concentration. The average height values obtained from three independent measurements were taken into account. Differential height is defined as  $[\Delta H]_p$  in the case of **D1-STV**, and  $[\Delta H]_{ab}$  in the case of **anti-STV IgG**. For **anti-STV IgG**, the measured differential height was corrected by subtracting the measured size of STV i.e.  $[\Delta H]_p$  to reflect the binding signal of **anti-STV IgG**. Assuming that the target-probe binding events are unaffected by surface coverage (i.e. the filled fraction of target molecules binding sites), and in addition, that the differential height is proportional to the surface coverage, Langumir equation states:

$$\Delta H = h \frac{[C]}{[C] + K_D}$$

where C represents the concentration of target molecules in solution (**D1-STV**, **anti STV IgG**), and h is a proportionality constant, which represents the saturation height signal at asymptotically high values of the concentration.

**Figure S1**

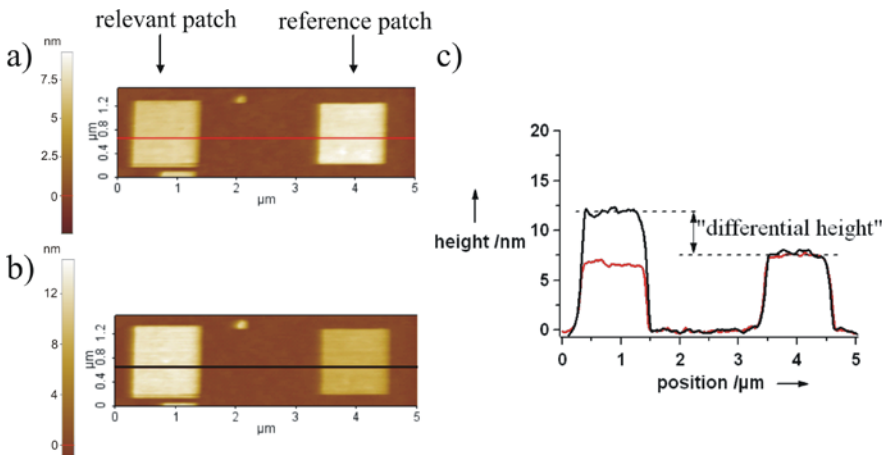


**Figure S1:** AFM topographic images ( $5 \times 1.5 \mu\text{m}^2$ ) of two ssDNA nanopatches indicated as relevant patch (cD1) and reference patch (cD2); a) before hybridization; b) after hybridization with D1-STV conjugate; c) corresponding height profiles with the description of “differential height”.

**Investigation of specific STV-biotin interaction.** Nanopatches of D1-STV were prepared as described above and exposed to  $1\mu\text{M}$  solution of biotinylated and non-biotinylated goat anti-mouse IgG to investigate whether the nanopatch-immobilized STV has retained its specific biotin-binding capability. The results are shown in Fig. S2.

**Figure S2**

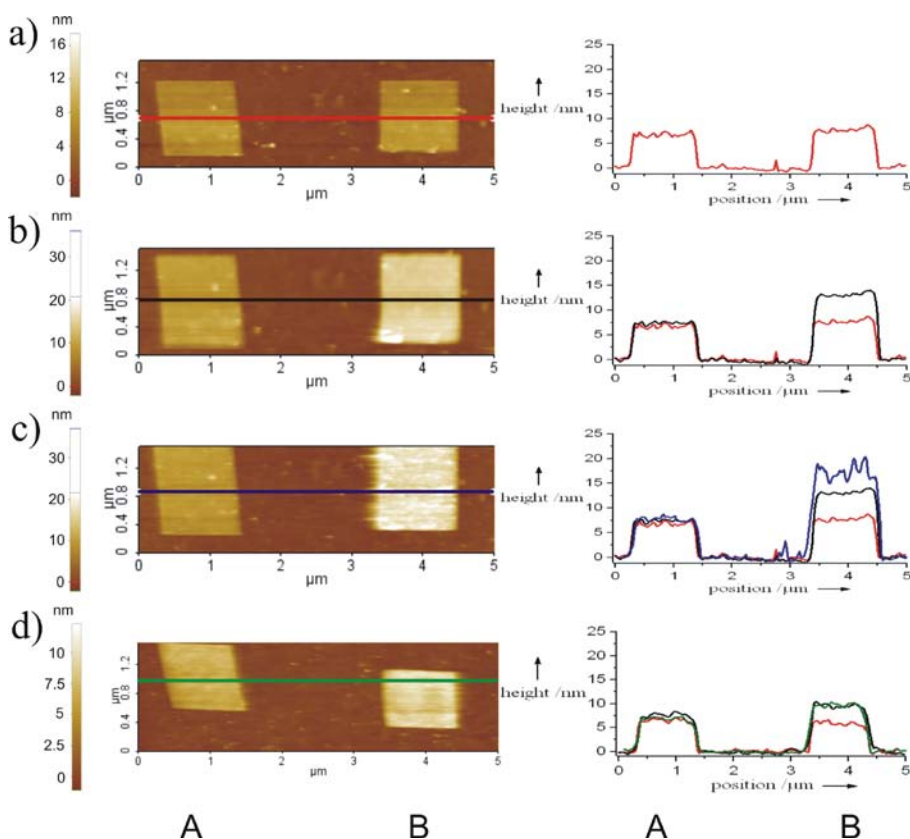


**Figure S2:** Interaction of D1-STV immobilized on a DNA nanopatch with a biotinylated antibody. Patches A and B contain non-complementary (control, cD0) and complementary capture oligomers (cD1) respectively. a) grafted ssDNA. b) D1-STV

immobilization, subsequent addition of c) biotinylated goat anti-mouse IgG and d) non-biotinylated goat anti-mouse IgG Comparison of c) and d) clearly indicates that only biotinylated IgG binds to the STV-modified patch.

**Scalability of nanografted patches.** Patches of ssDNA molecules (**cD1**) of various sizes, ranging from 200 x 200 to 1000 x 1000 nm<sup>2</sup> were prepared. Different S/A ratios were realized, ranging from S/A = 40 to S/A = 1. As already discussed, S/A is the ratio between the total area that is covered by the tip (S) during nanografting and the area of the patch (A) and is proportional to the density of the released ssDNA molecules in the nanopatch. Resulting AFM images and height profiles are shown in Fig. S3. In the height profiles, the red dotted lines indicate S/A = 1, while the green lines indicate S/A = 40. The sample was then incubated with DNA-protein conjugate (**D1-STV**, 2 μM, PBS), and height measurements were carried out. As shown in Fig. S3b, a clear increase in height of  $4 \pm 0.5$ nm was observed after hybridization of **D1-STV** conjugate for both S/A = 1 and S/A = 40.

**Figure S3:**

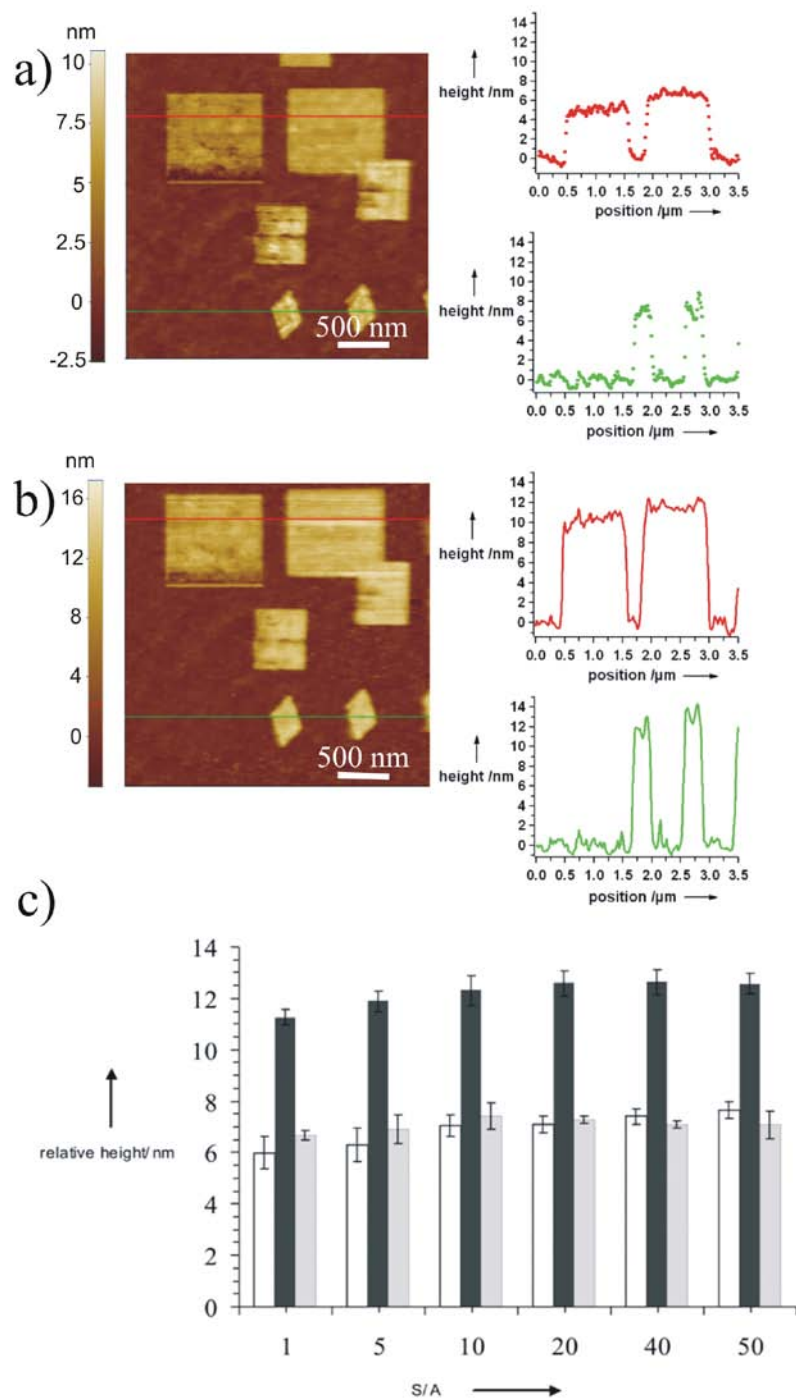


**Figure S3:** AFM topographic images ( $3.5 \times 3.5 \mu\text{m}^2$ ) of nano-patches of different sizes. a) the fabrication of ssDNA cD1 nanopatches by nanografting DNA in varying S/A ratios. The red and green lines indicate S/A=1 and S/A=40, respectively; b) hybridization of D1-STV conjugate and c) plot of relative heights of the patches as a function of S/A

before (white columns) and after hybridization with **D1-STV** conjugate (black columns). Grey column represent a control (hybridization of unmodified STV).

**Figure S4: DDI of D2-HRP and D1-GOx conjugates on DNA nanopatches and corresponding antibody binding.**

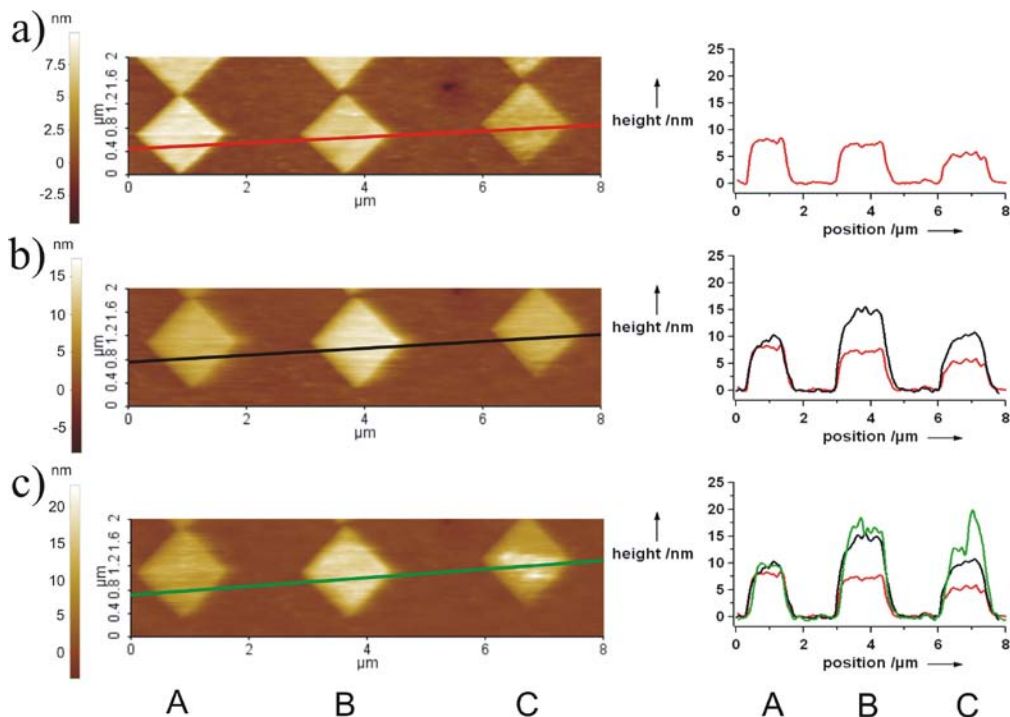


**Figure S4:** AFM topographic images ( $8 \times 2 \mu\text{m}^2$ ) and corresponding height profiles of antibody binding on the immobilized protein nanopatches; a) ssDNA nanopatches, **cD0** (control patch, A), **cD1** (patch B) and **cD2** (patch C), red line profile; b) patches after incubation with a solution containing DNA-protein conjugates **D1-GOx** and **D2-HRP**. Note that the increase in height indicates successful immobilization of both conjugates, **D1-GOx** (patch B) and **D2-HRP** (patch C), black line profile); c) patches after incubation with the mixture of **anti-HRP IgG** and **anti-STV IgG**. Note that only the HRP-containing patch (C) reveals an increased height (green line profile).

**Figure S5:** Glass microarray-based immunoassays to analyze possible cross reactions between DNA sequences, protein conjugates and antibodies.

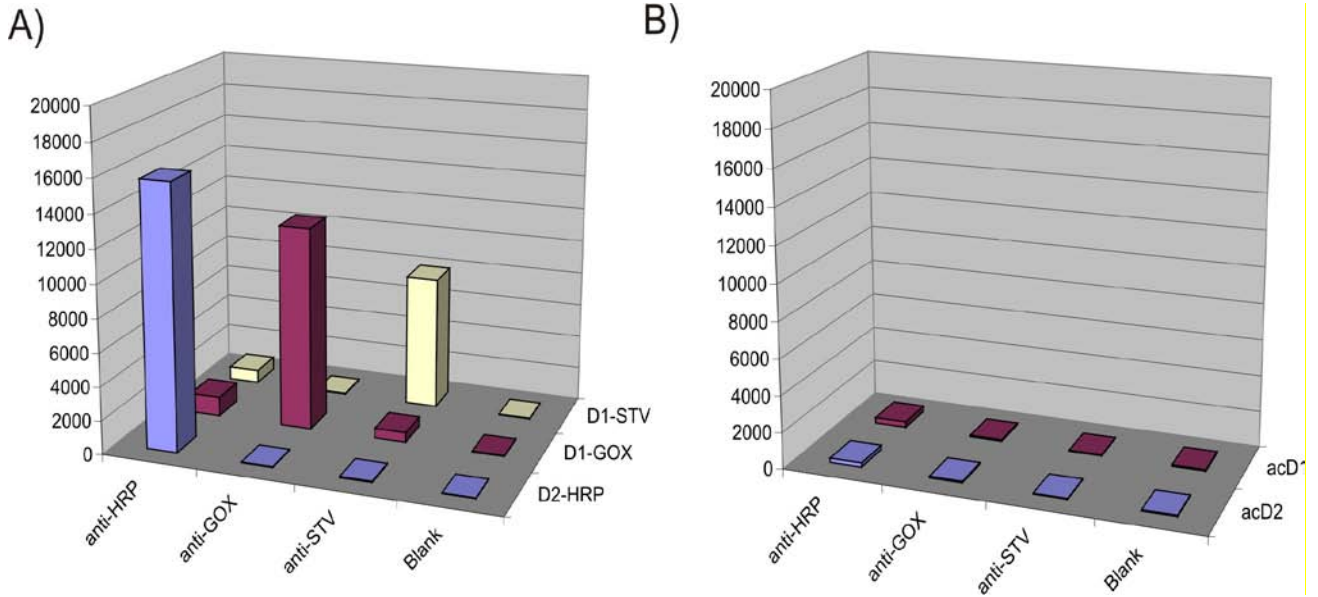


**Figure S5:** Immunoassay experiments performed on glass slides, using complementary DNA spotted as capture for DDI onto glass surfaces. A) DNA-protein conjugates (**D1-STV**, **D1-GOx** and **D2-HRP**) were hybridized to their complementary sequences and subsequently treated with specific fluorescently labelled antibodies (**anti-HRP IgG**, **anti-GOx IgG** and **anti-STV IgG**) B) Control slide where no proteins were hybridised.

**Experimental procedure.** To prepare the microarrays used for the micro-immunoassays, a solution of the desired 5'- amino-modified oligonucleotides (caD1 and caD2) was spotted onto 3DProtein Slides (Chimera Biotec, Dortmund, Germany) using a piezo-driven spotting device. The slides were incubated overnight and subsequently stored at 4 °C until use. The following incubation steps were carried out in a humidity chamber, under mild orbital shaking (100 rpm) at room temperature. Each incubation step was followed by washing with 60  $\mu$ L TETBS (2 x 30 s, 2 x 5 min). Prior to the first binding step, the spotted glass substrates were rinsed with TETBS, followed by blocking with MESTBS buffer for 30 min. Subsequently, the slides were washed, shacked dry and incubated with the specific DNA-Enzyme conjugates (D1-STV, D2-HRP, and D1-GOx) for 90 min. After washing, the substrates were incubated with 100 nM solutions of the primary antibodies (anti-STV, anti-HRP and anti-GOx all from rabbit) dissolved in standardized human serum (BISEKO). In the final incubation step, DyLight<sup>TM</sup> 549

conjugated anti-Rabbit IgG from goat was allowed to bind for 30 min (100 nM in TETBS). Final washing with TETBS and once with ddH<sub>2</sub>O was followed by centrifugation of the substrates at 1500 rpm for 3 min. Fluorescent signals on the glass slide was then analyzed using a GenePix 4100B Microarray Reader (Laser Power 100, PMTGain 330).

**Buffers used:**

|        |                                                                                                        |
|--------|--------------------------------------------------------------------------------------------------------|
| TETBS  | 20 mM Tris-Cl, 150 mM NaCl, 5 mM EDTA, 0.05% Tween-20, pH 7.5                                          |
| MESTBS | 20 mM Tris-Cl, 150 mM NaCl, 4.5 % milk powder, 5 mM EDTA, 0.2% NaN <sub>3</sub> , 1 mg/ml DNA, pH 7.35 |

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