Improving immunoassay performance with cleavable blocking of microarrays

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Supporting Information

Contents: quantification and characterization of vaccinia virus; contact angle measurements; nanoscale hydrophobicity studies; quantification of the fluorescence CT assay results on a logarithmic scale; effect of TCEP on immobilized antibodies; quantification of IL-1β assay results; calibration curve for CT assay on hydrophilic microarray; schematic of liquid draining from hydrophilic and hydrophobic microarrays; estimation of the thermodynamic sensitivity limit of immunoassay.

Quantification and characterization of vaccinia virus

The dry vaccine was dissolved in water and centrifuged at 3000 g for 5 min. Viruses were sedimented from the supernatant by ultracentrifugation at 30000 g for 1 h, resuspended in mQ water, applied onto a freshly cleaved mica surface, and dried in a stream of nitrogen. The sample was scanned using a SmartSPM^{TM}-1000 atomic force microscope (AIST-NT, Co, Moscow, Russia). In scanning experiments, the tapping mode with a resonance frequency of 100-150 kHz was used. A representative scan is shown in Fig. S1A. The average number of virus particles per unit area was calculated based on three scans of 20×20 μm^2, and from these data the concentration of the virus in the sample was estimated. The observed dimensions of an individual virus are 450×500×100 nm (Fig. S1B). The previously published dimensions of the dry vaccinia virus are 437×527×83.5 nm [S1], which is in good agreement with the observed dimensions of the virus used in our experiments.

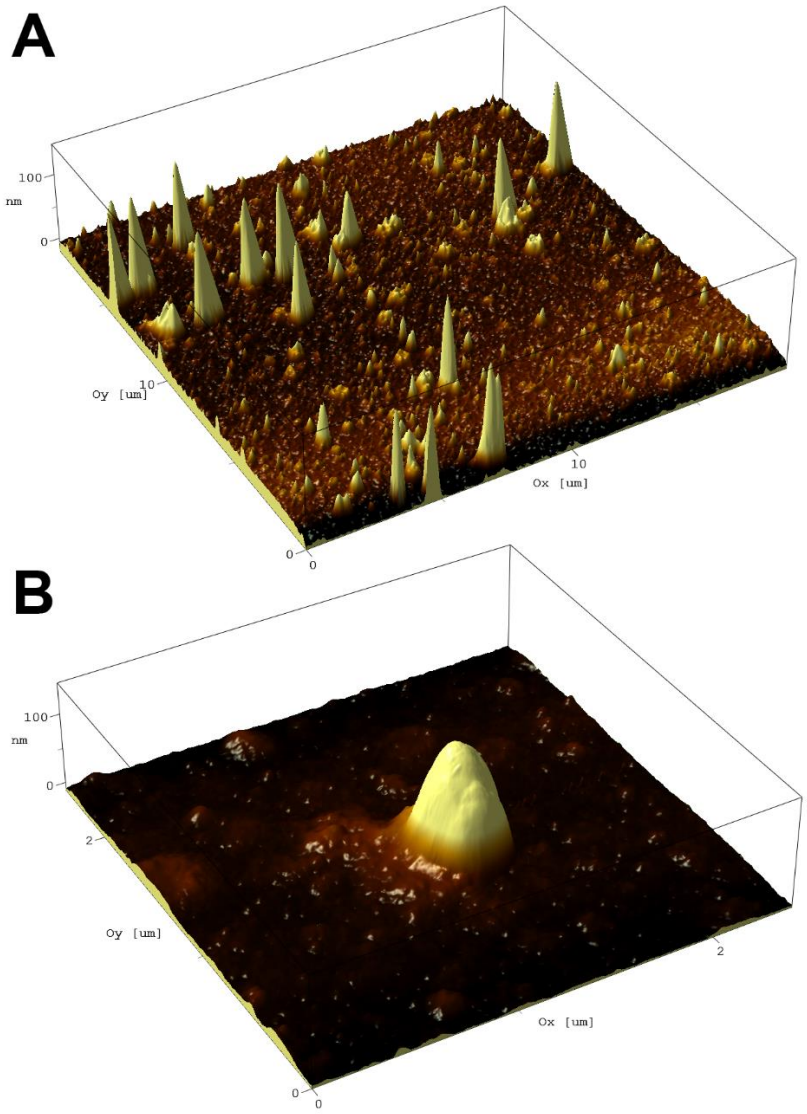

Fig. S1. Quantification and characterization of vaccinia virus by AFM.

Contact angle measurements

Efficient mass transfer of the analyte requires a large water contact angle, providing that no liquid layer remains on the microarray after draining the sample solution. Water droplets of 1 μL volume were placed onto the studied surfaces. Examples of the images obtained are shown in Fig. S2. To estimate the contact angle, at least 3 repeats of each experiment were used. The contact angle in the background zone, where the protein was not immobilized and which is expected to be densely covered with hydrophobic fluorinated groups, was 89 ± 15° (Fig. S2A). Since the microarray is supposed to be incubated in complex biological samples, the effect of possible protein adsorption on the contact angle was studied: it slightly decreased to 77 ± 17° (Fig. S2B) upon overnight incubation in human serum. The contact angle in active zones containing immobilized antibodies along with a fluorous coating was 70 ± 15° (Fig. S2C). It is lower than in the background zone, since some part of the surface is occupied by a protein that is less hydrophobic than a fluorous coating. Thus, the fluorous-blocked microarray surface is hydrophobic, and complete draining of aqueous solutions from it was observed. After TCEP treatment, the contact angle decreased both in the background and in the active zone. The corresponding values (14 ± 5°, Fig. S2D, and 16 ± 5°, Fig. S2E) were the same as for the native membrane (16 ± 7°, Fig. S2F), indicating complete removal of the fluorous coating.

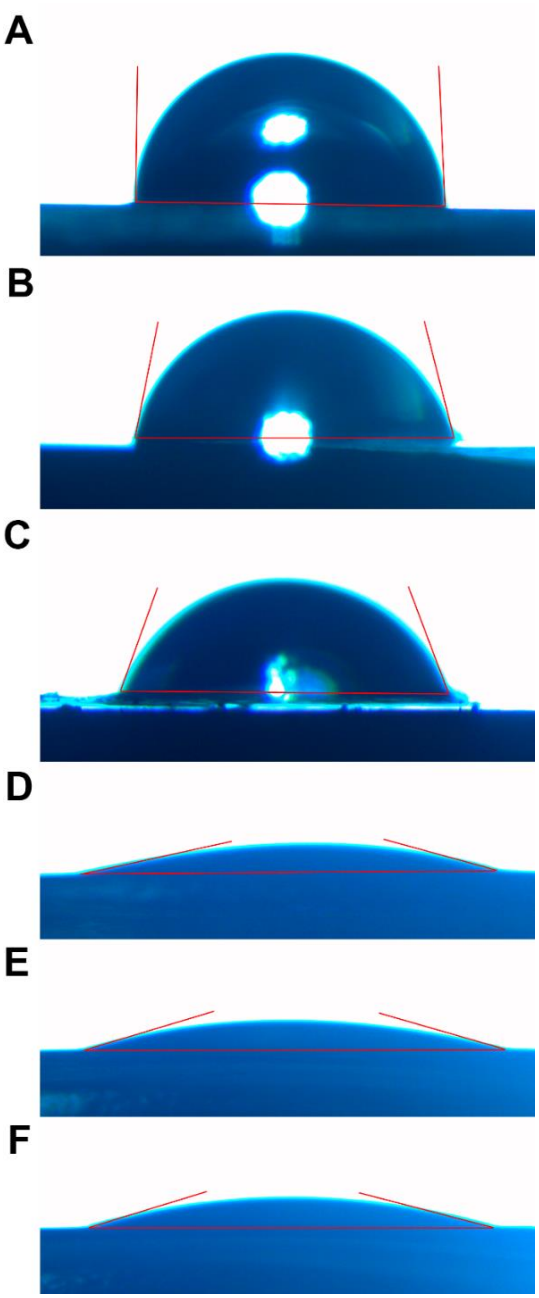

Fig. S2. Examples of water contact angles measured on different surfaces: A) background zone of fluorous microarray; B) background zone of the fluorous microarray after 12 h incubation with human serum; C) active zone of the fluorous microarray; D) background zone of the fluorous microarray after TCEP treatment; E) active zone of the fluorous microarray after TCEP treatment; F) native cellulose membrane.

Nanoscale hydrophobicity studies were carried out by measuring the adhesion force between the surface and the hydrophobic cantilever in water, as previously described [39]. Contact mode cantilevers fpC11 (NIIFP, Russia) with the resonant frequency of 15-20 kHz were hydrophobized by exposure to trimethylchlorosilane vapor for 1 h. Prior to the experiment, the sample and cantilever were equilibrated in water for 15 min. The contact mode was used to obtain 1×1 μm^2 scans of the studied surfaces. The force curve measurement was performed in 100 positions of each sample (Fig. S3). The adhesion force was calculated from the cantilever deflection value using its spring constant. Based on the obtained adhesion force values, the positions were grouped into high- (> 100 pN), medium- (10-100 pN), and low-adhesive (< 10 pN). Their spatial distribution and the occurrence frequencies for various investigated surfaces are shown in Fig. S3.

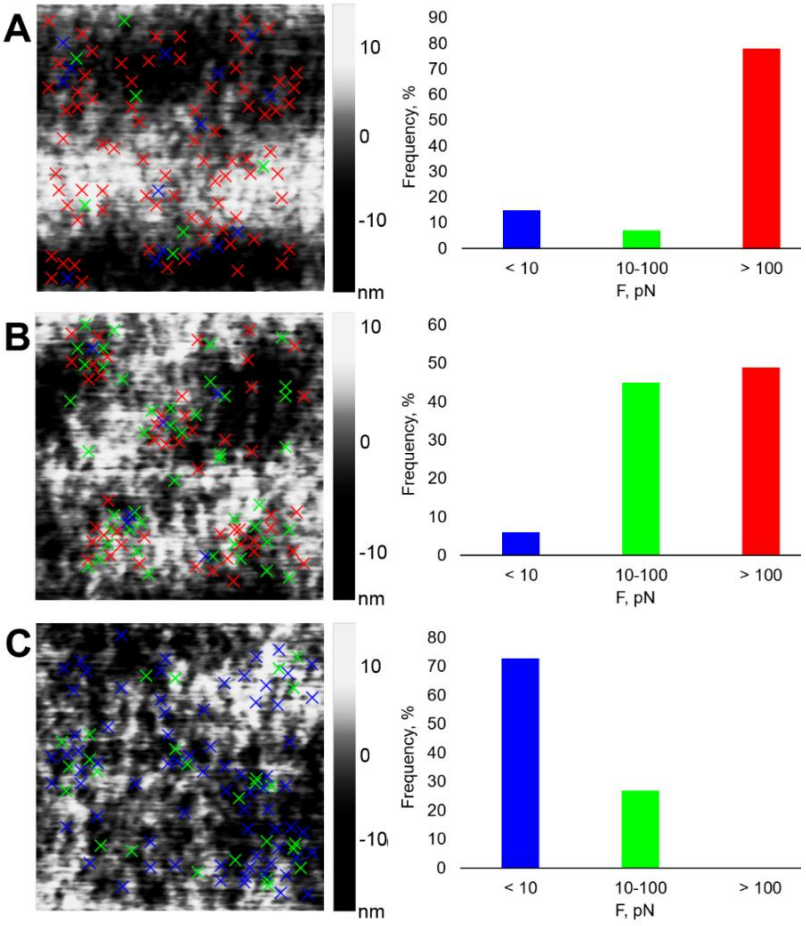

Fig. S3. The distribution of the adhesion forces of the hydrophobic AFM cantilever measured on different surfaces: A) the background zone of the fluorous microarray; B) the active zone of the fluorous microarray; C) the active zone of the fluorous microarray after TCEP treatment. In each case, 100 positions were analyzed. Red crosses correspond to high-adhesive sites (F > 100 pN), green crosses – to medium-adhesive ones (F = 10-100 pN), blue crosses – to low-adhesive ones (F < 10 pN). The scan dimensions were 1×1 μm^2. Histograms depicting the total number of high-, medium- and low-adhesive sites are shown on the right.

Quantification of the fluorescence CT assay results on a logarithmic scale

The same data as in Fig. 2B are presented on a logarithmic scale, showing also the autofluorescence of the substrates used (Fig. S4).

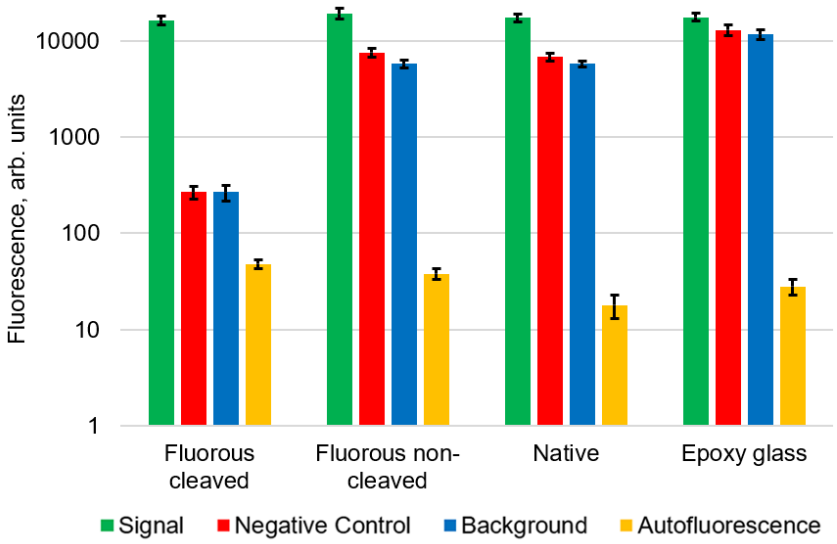

Fig. S4. Comparison of signal, negative control, background and autofluorescence values obtained in an immunofluorescent assay of a 100 ng/mL CT solution on a logarithmic scale.

Effect of TCEP on immobilized antibodies

Since the antibodies themselves contain disulfide bonds and thus can be cleaved by reducing agents, we further investigated the effect of TCEP treatment on the immunoassay performance. We performed a control experiment to detect CT using ethanolamine-blocked microarray. The experimental protocol was the same as described in the main text, except that the microarray was treated with TCEP (100 mM in 50 mM Tris-HCl, pH 8.0 for 5 min) either after binding of the analyte and detecting antibody, or prior to analyte binding. As seen in Fig. S5, in both cases only a slight signal decrease is observed, as compared to the control with no TCEP treatment. Antibodies were shown to be split by reducing agents into half-IgG fragments without significant loss of binding affinity [S2]. At the same time, splitting of binding antibodies can lead to signal loss in an immunofluorescent assay due to partial loss of bound analyte, while splitting detecting antibodies can lead to partial loss of a fluorescent label. However, this effect was observed only to a small extent, which may be due to several factors. The binding antibody can be tethered to the surface by multiple bonds, provided that the density of reactive groups at the protein immobilization step is high enough. Thus, when an antibody molecule is split, both halves of it remain covalently immobilized. Moreover, the duration of TCEP action is short is, and some sterically hindered disulfides can avoid reduction. In addition, even fully reduced detecting antibodies could be held together by non-covalent interactions for some time, which probably prevents elution of the fluorescent label, since the fluorescence detection is conducted immediately after TCEP treatment.

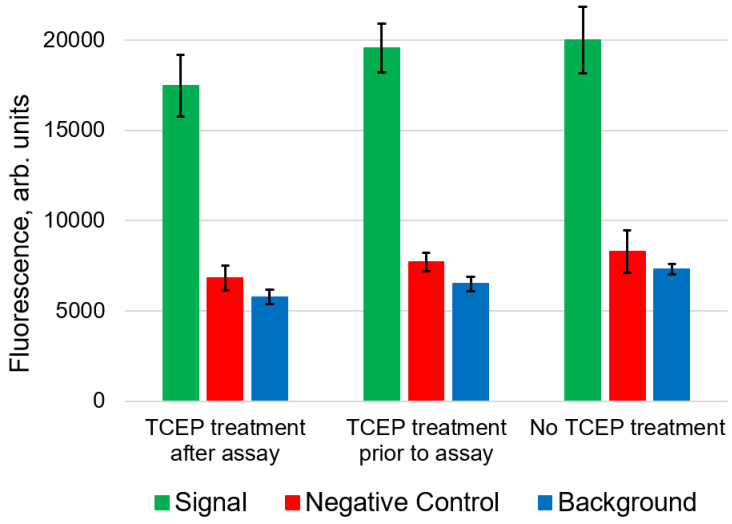

Fig. S5. Signal, negative control and background values obtained in immunofluorescent assays of a 100 ng/mL CT solution using ethanolamine-blocked microarrays either treated with TCEP after the binding of analyte and detecting antibody, treated with TCEP prior to analyte binding, or not treated with TCEP.

Quantification of IL-1β assay results

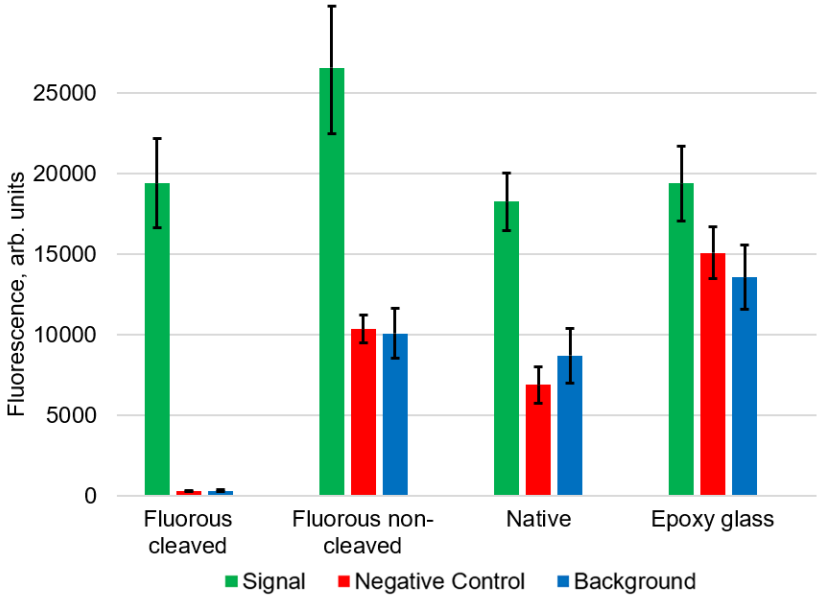

Fig. S6. Signal, negative control and background values obtained in immunofluorescent assay of a 100 ng/mL IL-1β solution using different microarray substrates: cellulose membrane blocked by a cleavable fluorous amine and cleaved by TCEP prior to detection; cellulose membrane blocked with a cleavable fluorous amine but not treated with TCEP prior to detection; cellulose membrane blocked with ethanolamine (native); a common epoxy-silane-coated glass slide.

Calibration curve for CT assay on hydrophilic microarray

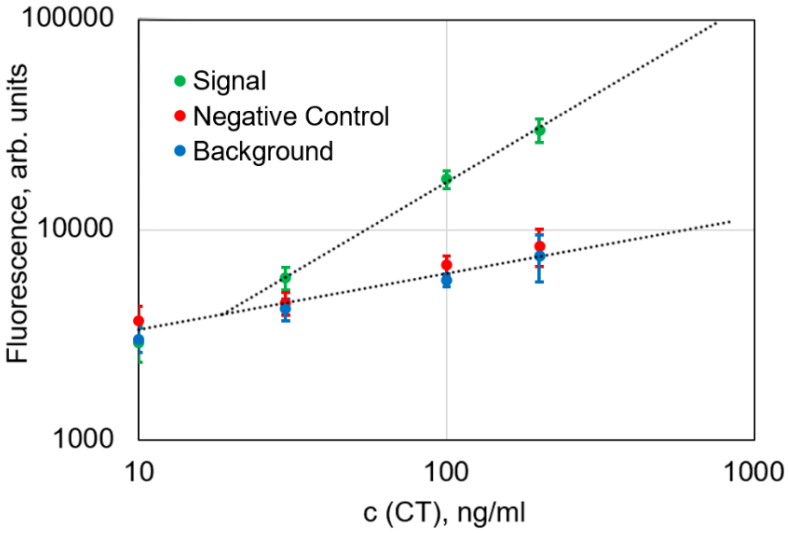

Fig. S7. Calibration curve for CT detection on an ethanolamine-blocked cellulose membrane-based microarray.

Schematic of liquid draining from hydrophilic and hydrophobic microarrays

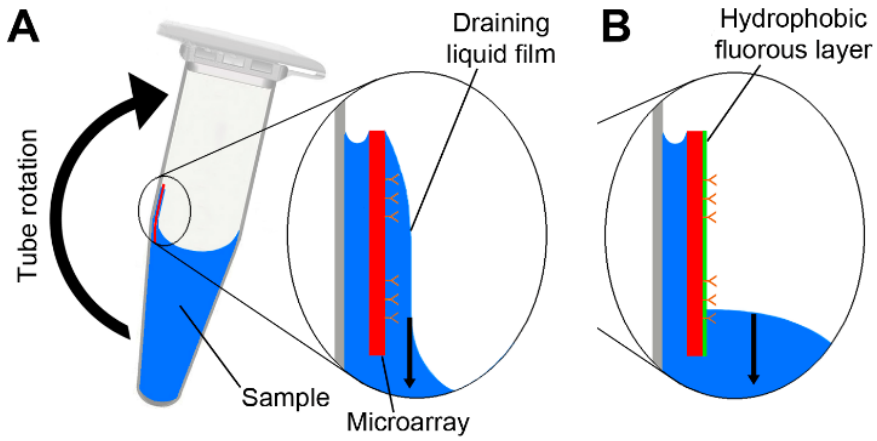

Fig. S8. Liquid draining from a membrane-based microarray (A) without and (B) with a hydrophobic coating in a rotating tube.

Estimation of the thermodynamic sensitivity limit of immunoassay

According to a static diffusion model [S3], the molecular flux into the microarray spot is:

$$J = 4DN_A ca \qquad (1),$$

where D is the diffusion coefficient of the analyte (10^{-6} cm^2/s for a medium-sized protein), c is its concentration, and a is the microarray spot radius (50 μm). As shown in Fig. 3B, a minimal detectable signal of 3-5 beads per spot is accumulated in 10-20 min of static incubation in 10 fM CT solution. The corresponding diffusive flux of analyte (1) is ~1 molecule in 10 s or ~100 molecules in 20 min. Thus, to obtain a minimal detectable signal, one needs ~100 molecules to be captured on the microarray spot. If the typical value of immobilization density of antibodies, Γ, is ~ 10^4 molecules per μm^2 [S4], we have 10^8 binding centers per 100 × 100 μm^2 spot. The equilibrium constant of affinity of binding antibody to CT was measured previously [31]:

$$K = [\text{Ab}\cdot\text{CT}]_S/[\text{Ab}]_S\,[\text{CT}] \sim 10^9 \qquad (2),$$

where subindex S denotes the surface concentration. Thus, the minimal detectable equilibrium analyte concentration is $[\text{CT}] \sim [\text{Ab}\cdot\text{CT}]_S/K/[\text{Ab}]_S = 100/10^9/10^8 = 1$ fM, which is consistent with the experimentally observed LOD.

References

S1. Johnson, L.; Gupta, A. K.; Ghafoor, A.; Akin, D.; Bashir, R. Characterization of vaccinia virus particles using microscale silicon cantilever resonators and atomic force microscopy. *Sensors and Actuators B: Chem.* **2006**. *115*, 189–197.

S2. Karyakin, A. A.; Presnova, G. V.; Rubtsova, M. Y.; Egorov, A. M. Oriented Immobilization of Antibodies onto the Gold Surfaces via Their Native Thiol Groups. *Anal. Chem.* **2000,** *72*, 3805–3811.

S3. Sheehan, P. E.; Whitman, L. J. Detection Limits for Nanoscale Biosensors. *Nano Lett*. **2005**, *5*, 803–807.

S4. Ahluwalia, A.; De Rossi, D.; Ristori, C.; Schirone, A.; Serra, G. A comparative study of protein immobilization techniques for optical immunosensors. *Biosens. Bioelectron.* **1992**, *7*, 207–214.