Electronic Supplementary Information

Controlling the immobilization process of an optical enhanced protein microarray for highly reproducible immunoassay

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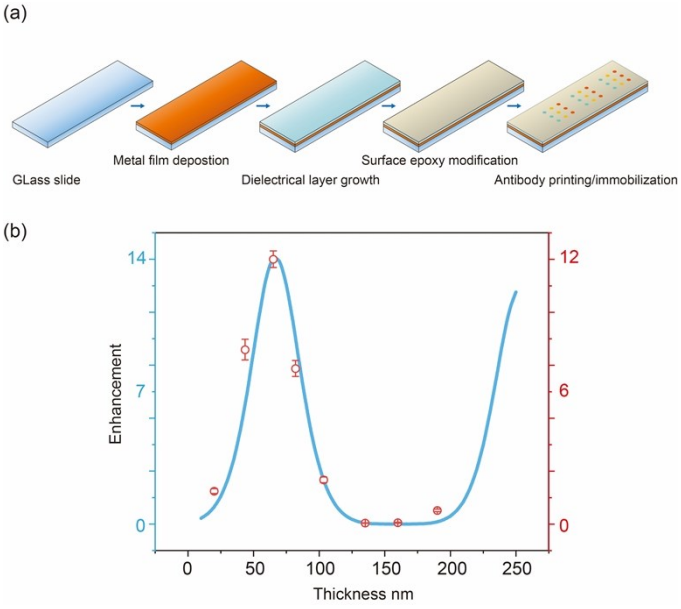

Figure S1 (a) The illustration of the fabrication process of OEPM. **(b)** Experiment (red circle) and simulation (blue curve) results of enhancement with different Al_2O_3 thickness on OEPM.

1. FABRICATION, EXPERIMENT, AND SIMULATION OF OEPM.

There are two ways to achieve optical interference on the surface. First is the Bragg mirror, which using two transparent materials with a large difference in refractive index, and they are deposited alternatively on the surface multiple times. Light could be reflected between the interface of these materials and interference. The second method utilizes a highly reflective material, normally metal film such as aluminum, silver, or gold, under a thin transparent layer. The light would be largely reflected by the metal film and interference on the transparent layer surface. Although the Bragg

mirror is more powerful in controlling the interference for different wavelengths, such as multi-band optical filters, the second method is more preferred for OEPM since it's simple and effective for fluorescence. The illustration of the OEPM fabrication process is shown in Fig. S1 (a). A silver layer was deposited on the glass slide as a high reflective layer, then a transparent dielectric layer was grown onto the silver to act as an interference layer. There are several ways to grow the dielectric layer, such as atomic layer deposition (ALD), electron beam vapor deposition (EB-PVD), and magnetic sputtering (MS), and chemical method. The diversity of the growing method offers convenience for more complex structures such as plasmon nanostructures.

Besides SiO_2, OEPM with different Al_2O_3 thickness ranging from 20 – 200 nm were fabricated as well. Immunoassay with mouse IgG as the antigen was performed on Al_2O_3 OEPM. The enhancement times of Al_2O_3 OEPM was obtained by dividing the signal on the glass. The comparison of experiment and simulation was shown in Fig. S1(b). The maximum enhancement appeared around 70 nm for simulation with 14.1 times, and 65 nm for the experiment about 12 times. The destructive interference showed around 150 nm. Due to the time costing in ALD growth, Al_2O_3 film was not applied to OEPM. The simulation showed that, although the refractive index is different, the maximum enhancement is similar. Also, the experiment shows the best enhancement around 12 times.

2. INFLUENCE OF CONCENTRATION, SDS, WASHING AND DRYING ON SMEARING AND REPRODUCIBILITY

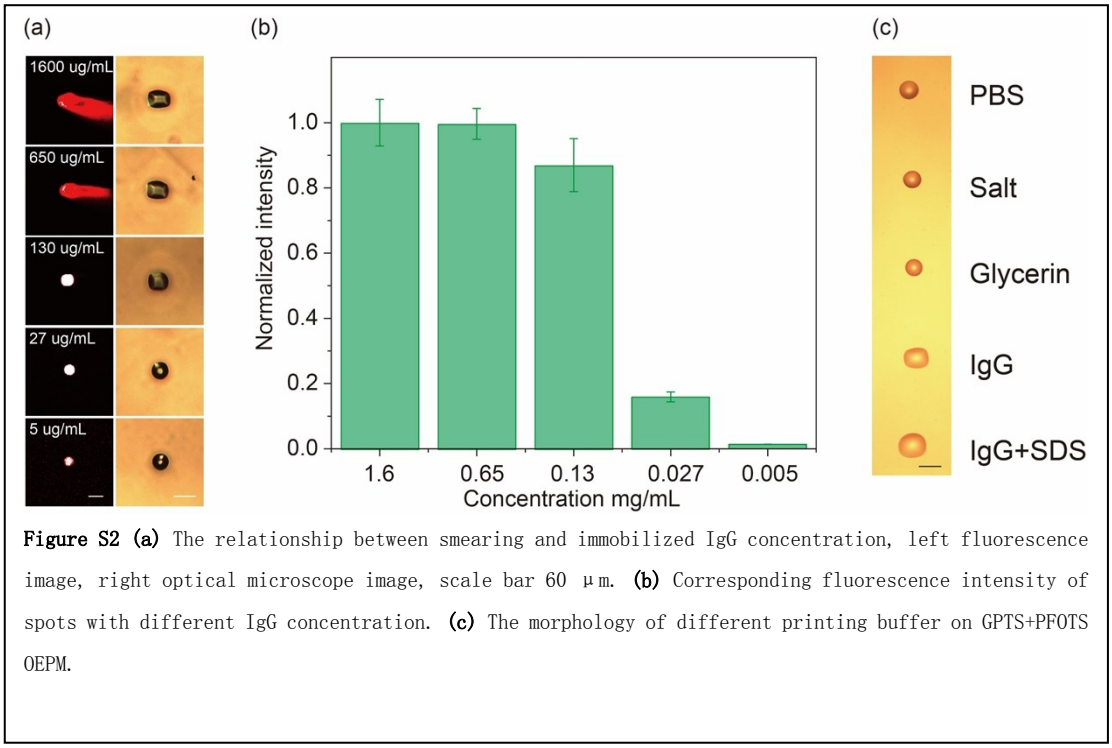

Figure S2 (a) The relationship between smearing and immobilized IgG concentration, left fluorescence image, right optical microscope image, scale bar 60 μm. **(b)** Corresponding fluorescence intensity of spots with different IgG concentration. **(c)** The morphology of different printing buffer on GPTS+PFOTS OEPM.

The relationship between the printing concentration of IgG and smearing is obvious, however, we could not easily conclude that the smearing comes from excessive IgG adsorbed on the unwanted area. Except for smearing, the shape of spots changed enormously from square to round, as shown in Fig. S2(a), which is believed to under the control of wetting ability. How the IgG concentration changed the spots' shape is not understood. Also, the signal gain from increasing immobilization concertation is saturated around 0.65 ug/mL as shown in Fig. S2(b), higher concentration no longer improves the signal intensity. Thus, in the main text, we focused on optimization under 0.65ug/mL. To identify which component, influence the shape, a series of printing buffer were prepared, the results are shown in Fig. S2(c). The base component of the printing buffer contains 25% glycerin, 25% 1.5 M ammonium sulfate solution, the rest 50% were adjusted differently with PBS, 1.5 M ammonium sulfate, glycerin, IgG, and IgG+SDS. As we can see, only buffer with IgG will show square shape, others keep rounded. Thus, the square shape must come from the influence of the IgG. Unlike small surfactants, the increasing concertation will improve the wetting ability, IgG itself is a kind of large molecule with hydrophobic and hydrophilic groups, and increasing the IgG concertation seems to reduce the wetting ability while. The huge difference in wetting behavior with concentration indicating the complex interaction of IgG with itself and other molecules.

The washing process is another factor for smearing generation, but there are different perspectives on using quick or slow washing. Commercial protein microarray products, such as GenTel from BioSurfaces and Intuitive from BioSciences, recommend quick washing and blocking process in the protocols to prevent smearing. However, some groups suggest slow washing to avoid smearing as mention in the main text. Different washing speed was tested on the smearing generation of OEPM modified with GPTS+PFOTS OEPM, as shown in Fig. S3(a). We can see that washing speed

has a limited impact on smearing generation, but it influences the length of the tail to some extent. The controlled washing process worked well for all GPTS modified substrates. However, when quickly immersed into the wash buffer, the spots showed strong smearing with GPTS OEPM as shown in Fig. S3(b). This is contrary to commercial protocols.

Rather, an extremely slow washing speed reduces the signal and increases the standard deviation significantly. We plotted the signal along the washing direction of each spot under 2 μm/s washing speed, as shown in Fig. S3(c), there shows an increase in signal intensity. Although the spots signal was different from one to another, the intensity distribution inside the spots was uniform. Another nonuniformity is showed in Fig. S3 (d), where the drying pattern overlapped with spots. The overlapping region, encapsulated by the blue dash line, is darker than other regions and other spots. This strongly indicates that the desorption happened during the drying rather than deactivation. Why the image in Fig. S3(d) showed an apparent defect, a broken spot, but the image in Fig. S3 (c) didn't? We think the slow washing process made the desorption or deactivation gradually over the whole spot, but the drying process during the assay was not controlled, the evaporating of tiny drops induced flow was sudden and strong.

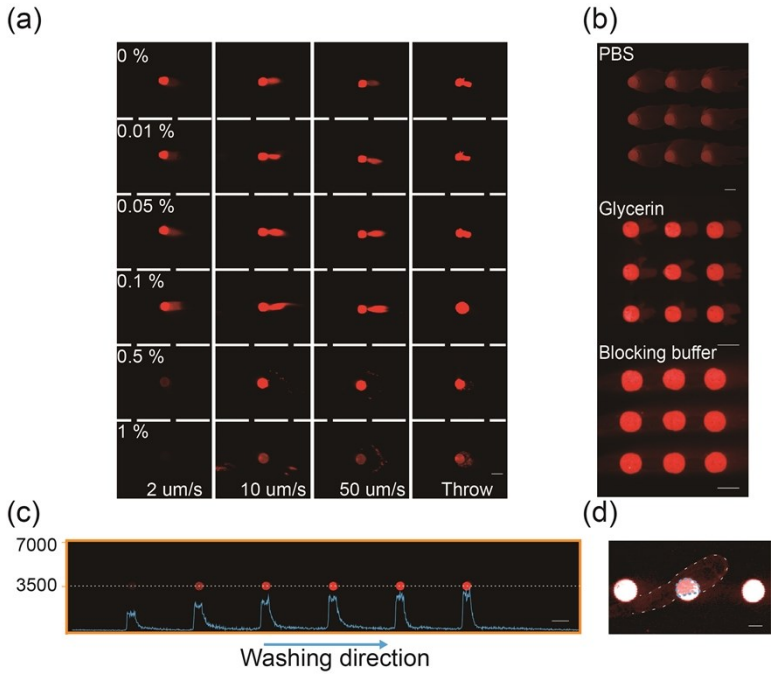

Figure S3 **(a)**Different washing speed on GPTS+PFOTS modified OEPM with printing buffer containing various amount of SDS. **(b)** Fluorescence images of spots smearing resulting from quick immersing the GPTS modified OEPM into different washing buffer. **(c)**Intensity variation under low-speed washing. **(d)**The drying pattern (white dash line) overlapping (red dash line) with immobilized spots. The scale bar is 90 μm.

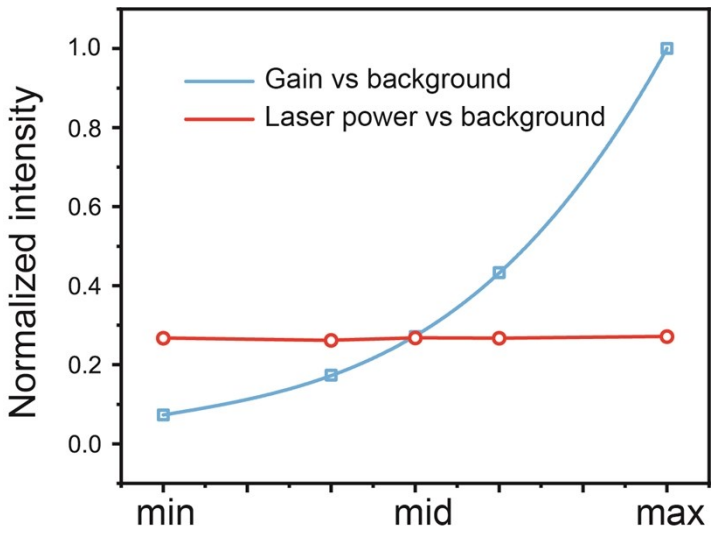

Figure S4 The relationship between PMT gain and background, blue line. The relationship between laser power and background, red line. The laser power and gain share the same condition in the middle point

3. THE INFLUENCE OF PMT GAIN AND LASER POWER ON BACKGROUND/BLANK OF OEPM.

The sensitivity or limit of detection is 3 times of background or blank signal on the concentration curve. To improve the LOD, a smaller background is preferred. However, for PMT gain and laser power, their influence on the background is different. This is because the origin of the background for each parameter is different. For PMT gain, the background mainly comes from the noise of the instrument, which could be amplified by giving larger feedback in PMT (higher gain). On the other hand, laser power has little effect on this noise. The main contribution of the background for the laser is the unspecific adsorption of the fluorescence molecule, and this could be solved by efficient blocking after immobilization. The influence of gain and laser power on the background of OEPM is shown in Fig. S4. The blue curve showed an exponential increase as gain approaching max, however, the background barely moved as laser power changed from min to max. The laser power and gain share the same condition in the middle point.