Supporting Information

Transmissive Nanohole Arrays for Massively-Parallel Optical Biosensing

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Fabrication of Nanohole Arrays

A 500-nm-thick layer of positive photoresist S1805 (Shipley) on a glass slide was patterned by a Lloyd’s mirror-based interference lithography system with a 363.8-nm argon ion (Ar⁺) laser. The period of interference fringes is tunable from 200 nm to 2,000 nm by adjusting the angle of the incident laser beams. The duration for a single exposure is ~1 to 3 min depending on the interference period. Two-dimensional arrays were created by double exposures with the second exposure at a 90-degree rotation relative to the first exposure. The developed pattern was coated with 30-nm Cr as an adhesion layer followed by 30-nm Au using electron beam evaporation. Lift-off of the S1805 photoresist in acetone resulted in a 60-nm thick opaque film containing a 2D array of nanoholes on the glass substrate. The nanohole array substrates (which could be as large as 10 cm in diameter) were cut into 5 × 5 mm pieces from a 25 × 25 mm glass substrate for further functionalization and testing.

It should be mentioned that nanohole arrays can vary in size and shape from batch to batch, mainly due to variations in UV exposure dose or time. These variations can be seen in the SEM images in Fig. 2-3 and Fig. S1 below. Such variation will not affect the reliability of the detection, however, as
long as nanoholes in the same array or batch are relatively uniform. This uniformity is further supported by Fig. S1-2, in which nanohole brightness is plotted along a single-pixel line. The brightness of the holes in a given chip is very uniform until they are blocked, even partially, with silver precipitate.

Figure S1. (a-c) Optical and SEM images of nanohole arrays with slightly different shapes and sizes. (d) Plots of nanohole brightness across the highlighted lines in (a-c). Curves are offset for easier viewing.
Figure S2. Plot of brightness along selected holes in Fig. 3. Note that even if the silver precipitate is less than half the size of the hole, a significant reduction in transmission is observed (labeled with blue stars).

**Biofunctionalization of Nanohole Sensors with Antibodies**

The selective functionalization and bioconjugation protocol is shown schematically in Fig. S3. The nanohole arrays were treated with oxygen plasma (Oxford Plasmalab 80 Plus, 50 W, 20 sccm O₂ flow rate) immediately prior to functionalization to remove organic residues from the surface introduced during the lithography and lift-off processes, and to increase the hydrophilicity of the glass by maximizing coverage of surface hydroxyl groups. The bare glass surface inside the holes was functionalized with aldehydes using triethoxysilylbutyraldehyde (TESBA, Gelest Inc, 2% v/v in 95% ethanol/5% DIW), and then the Au surface between holes was passivated with a self-assembled monolayer (SAM) of thiol-poly(ethylene glycol) (HS-PEG, NANOCS, Inc., 5 mg/mL solution of
1,000 g/mol in 95% ethanol/5% DIW). Murine monoclonal HyHEL-5 anti-lysozyme antibodies (HyHEL-5 custom produced by Biovest International Inc., 100µg/mL) were covalently attached to the glass in the nanoholes by reductive amination with the TESBA surface aldehydes in the presence of 50 mM sodium cyanoborohydride (Thermo Scientific). Selective functionalization reduces non-specific adsorption of analyte on the Au surface, and confines the analyte binding events inside the nanoholes.

Detection of Biotinylated Lysozyme with Silver Staining
Lysozyme from chicken egg white (Sigma-Aldrich) was biotinylated with EZ-Link sulfo-NHS-LC-biotin (Thermo Scientific, Product # 21335). The degree of biotinylation was approximately 1 biotin group per lysozyme molecule as determined by a HABA assay in a microplate format with absorbance measurements taken with a Tecan Infinite M200 Pro. Serial dilutions of biotinylated lysozyme (biotin-HEL) with concentrations ranging from 100 pg/mL to 10 µg/mL were prepared in PBS with 1 mg/mL of BSA added as sacrificial passivating protein. Negative controls consisted of 1 mg/mL BSA in PBS without biotin-HEL. After incubating with the analyte (samples were immersed in the analyte solution overnight at 4 ºC), samples were incubated for 1 hour at 37 ºC with Streptavidin poly-horseradish peroxidase 80 (SV-polyHRP, Fitzgerald) which served as the enzymatic reporter. Silver staining was carried out using the Nanoprobes Inc. EnzMet kit (Product # 6010) according to the manufacturer’s protocol. After 6 min incubation, the samples were washed several times with DI water to stop the silver staining reaction, and then the samples were dried under nitrogen before imaging. Optical detection was performed directly on dried samples without using cover slips.
Optical Image Processing and Analysis

Optical microscopy images of nanohole samples were captured by a microscope equipped with a 50× long working distance objective (Numerical Aperture = 0.55) and a color digital camera. The system can operate in both reflection mode and transmission mode. For clarity of presentation, all optical images shown in this paper were cropped and converted to 16 bit gray value images using Photoshop (Adobe). Image analysis can be carried out simply with most standard software tools. In the following, we show how the open source software ImageJ (http://rsb.info.nih.gov/ij/) can be used to count the number of blocked nanoholes. First, the brightness is plotted using the function “Plot Profile.” Then, images before functionalization (“Before”) and after silver staining (“After”) from the same position are processed as follows: (1) the images are digitized using the “Threshold” function, (2) the images are then reversed by choosing “Dark background” (holes become dark spots), and (3) the number of visible holes is counted using the “Analyze Particles” function (Fig S4).
Figure S4. Image processing using ImageJ for a pair of “before” and “after” images with biotin-HEL concentration of 100 ng/mL.