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Label-free and high-throughput detection of biomolecular interactions using a flatbed scanner biosensor

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KEYWORDS DNA/protein microarray; label-free; high-throughput assay; microarray calibration; low-cost biosensor.

ABSTRACT: Fluorescence based microarray detection systems provide sensitive measurements, however variation of probe immobilization and poor repeatability negatively affect the final readout, and thus quantification capability of these systems. Here, we demonstrate a label-free and high-throughput optical biosensor that can be utilized for calibration of fluorescence microarrays. The sensor employs a commercial flatbed scanner, and we demonstrate transformation of this low cost (~100 USD) system into an Interferometric Reflectance Imaging Sensor through hardware and software modifications. Using this sensor, we report detection of DNA hybridization and DNA directed antibody immobilization on label-free microarrays with a noise floor of ~30 pg/mm², and a scan speed of 5 seconds (50 seconds for 10 frames averaged) for a 2 mm x 2 mm area. This novel system may be used as a standalone label-free sensor especially in low-resource settings, as well as for quality control and calibration of microarrays in existing fluorescence-based DNA and protein detection platforms.

Biosensors that enable detection of DNA-protein, DNA-DNA or antigen-antibody interactions have been crucial in molecular diagnostics for the detection and monitoring of diseases. For low resource settings and point-of-care applications, the demand for cost-effective, easy-to-use, portable, high-throughput and sensitive biosensors is ever increasing.^{1,2} A commonly preferred technique, fluorescence microarrays, provides high sensitivity and throughput levels, however the detection of molecular interactions relies on secondary probes, which increases the complexity of these assays, and requires trained personnel and laboratory environments.

Optical label-free detection techniques, where changes in optical signals are monitored without the need for secondary probes, offer unique opportunities by reducing the complexity of the assay without necessarily compromising on the high-throughput.^{3,4} However, for microarray applications, they have yet to meet the sensitivity levels achieved by fluorescence based detection methods.

Besides complexity and high cost, fluorescence based detection methods suffer from another limitation: providing reliable quantitative results. To detect analytes on surface, these methods rely on immobilization of probes in a microarray format, which has been shown to be rather variable, especially for

protein microarrays.^{5,6} This has been considered a significant obstacle in the utilization of protein microarrays in clinical use. Without true quantification and accounting for the variation of the amount of initial probes and changes introduced on the microarray at every step of the assay, the interpretation of the results is misleading.^{7,8} Several techniques have been proposed to quantify initial probe immobilization, including both labeled and label-free procedures.⁹ Labeled techniques can only provide an indirect measurement of the surface bound analytes. They are often used to only test a representative set of later discarded microarrays within a batch of similarly produced arrays in order not to alter the binding kinetics by introducing labels. Label-free methods such as those based on surface plasmon resonance,¹⁰ photonic crystals,^{11,12} and reflectance interference spectroscopy^{13,14} have been suggested for quantitative detection of initial probe density. Among these Interferometric Reflectance Imaging Sensor (IRIS), offers easy adaptation for use with fluorescence microarrays, as it uses a silicon oxide on silicon surface, which is compatible with fluorescence based surface preparation and measurement protocols. A method based on this detection modality has been previously introduced for the calibration of fluorescence microarrays.¹⁵

Here, we introduce a label-free optical biosensor that is based on the principles of reflectance interference spectroscopy. Our optical system benefits from technological advancements in commercial electronics to reduce the hardware cost to as low as 100 USD. In the last decade, with the advances in consumer electronics, compact, mobile, cost-effective and high performance devices have been made available to end users, which led to an increased interest in their integration into scientific sensing and imaging platforms.¹⁶ Mobile phones,^{17–22} wearable computers,²³ and document scanners²⁴ have been converted to biosensing platforms with simple modifications. In particular, flatbed scanners, which are mainly designed for document scanning, offer large field of views (FOV), of the order of the size of an A4 paper, and high spatial resolutions of $\sim 10\ \mu\text{m}$ at an extremely low cost. Here, we employ a contact image sensor (CIS) type scanner, where the sample is illuminated sequentially with red, green and blue LEDs, and the scattered light is focused to an optoelectronic detector by a GRIN lens array. Through hardware and software modifications, we demonstrate the adaptation of a scanner as a label-free biosensor, and provide comparisons of this newly developed sensor with a conventional IRIS for the analysis of DNA microarrays.

METHODS

Interferometric Detection. IRIS utilizes the spectral reflectance signatures from a layered sensor chip. The sensor surface is thermally grown SiO_2 layer on Si, and any biomass accumulated on top of the oxide layer creates an optical path difference (OPD) between the top of the biomass layer and the SiO_2 -Si interface. The reflectivity of the substrate can be expressed by Fresnel equations and is given by

$$R = \frac{r_1^2 + r_2^2 + 2r_1r_2 \cos 2\phi}{1 + r_1^2 + r_2^2 + 2r_1r_2 \cos 2\phi} \quad (1)$$

where r_1 and r_2 are the Fresnel reflection coefficients for the air- SiO_2 and SiO_2 -Si interfaces respectively and ϕ is the OPD given by

$$\phi = \frac{2\pi t}{\lambda} n_{\text{ox}} \cos \theta \quad (2)$$

where t is the total thickness of the oxide layer, λ is the illumination wavelength, n_{ox} is the refractive index of SiO_2 , and θ is the angle of incidence. Any increase in the thickness of the top layer due to biomass accumulation, introduces an OPD, resulting in a shift in the spectral reflectivity curve. The reflectivity curve can be sampled at different wavelengths by changing the color of the incident light, and the thickness t can be calculated for each pixel in the image by minimizing the error when solving Eqn. (1) and (2).

Scanner Modifications. The hardware configuration for document scanning is based on the collection of scattered light from paper surface by graded-index (GRIN) lenses. In this case, image is acquired in a dark-field configuration, where

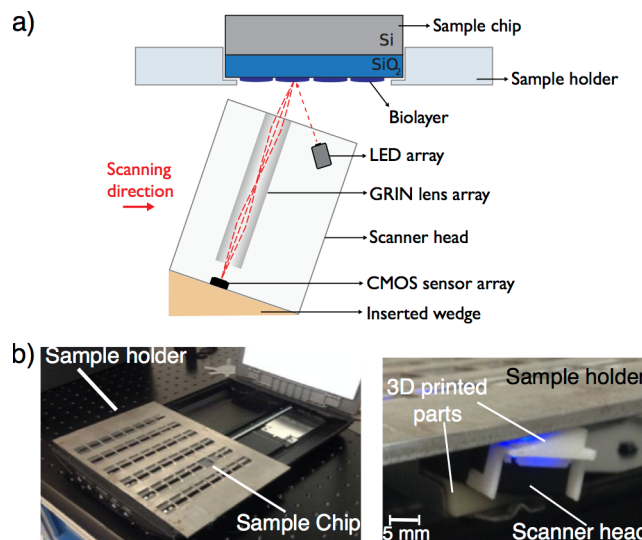


Figure 1 Modified flatbed scanner biosensor. (a) Schematic view of the scanning head and sample cross-section. (b) Experimental setup is a modified commercial flatbed scanner. Top view is shown on the left. Modifications include inserting a sample holder and re-aligning scanning head (right).

specular reflection from the object does not reach the optical sensor. In contrast, the sensor principle we adapted is based on detection of reflection from the sensor surface, thus it requires bright-field imaging. Therefore, in order to convert the document scanner to an interferometric sensor, the scanning head is tilted to collect the reflected light by inserting a 3D printed wedge underneath as shown in Fig. 1a. The scanner glass, which holds the document to be scanned, is replaced with a custom sample holder (Fig. 1b), which houses through holes for holding the sensor chips above the scanner head. The through holes of the custom-made holder are at varying heights with $250\ \mu\text{m}$ steps to provide optimization for best focus, as the depth of field of GRIN lenses is low (Fig. S-1) in comparison to the microscope objectives used in a conventional IRIS setup. In addition, the sample holder serves as a weight to keep the scanning head stable during the scan.

To overcome the restrictions of the commercially available acquisition software, we used an open source application programming interface (API) package called Scanner Access Now Easy (SANE). In this way, the central controller of the Canoscan LIDE 210, Genesys Logic GL124 can be programmed. Using this API, LEDs can be turned on and off individually, and their intensities can be adjusted. The scanning area, which is restricted in the commercial software for highest resolution settings can also be adjusted through the API. As we replaced the scanner glass with a custom holder, the extra calibration step, where the head scans a white stripe on the scanner glass prior to each scan to set individual pixel gain, was eliminated.

Data Acquisition and Processing. In this work, instead of fitting each pixel intensity to a corresponding thickness value for every image, we implemented a lookup table approach as explained in recent work to decrease computation time and complexity of data processing.²⁵ Briefly, images are captured by illuminating the chip with different colored LEDs. Reflectivity value of the SiO₂ reference region is calculated for each color by averaging corresponding pixel values within the selected region. A reflection curve is fitted to these values by solving Eqn. (1) and Eqn. (2) while minimizing the error. By using this fitted curve, a reflectivity corresponding to each thickness value, is calculated. Once this lookup table (LUT) is generated, for all following measurements, the pixels on the captured images can be easily mapped to a thickness value. We first generated this lookup table for the conventional IRIS setup (Fig. S2). Then, after calibration of the modified scanner using IRIS, we used the generated look-up table for all subsequent scanner measurements.

For scanner measurements, the scanning resolution and the bit depth are set to 4800 dpi and 16 bit, respectively. Scanning is done in color mode; however, analysis was performed using green channel images. Red channel images are used to generate a mask to eliminate alignment marks for spot analysis (Fig S3). Prior to analysis, 10 frames are averaged. In captured raw images, fringes were observed perpendicular to scanning direction as shown in Fig. 2, which are attributed to the GRIN lens array in the collection path. These fringes are repeatable in the scanning direction, and can be removed by dividing the raw image by a reference image obtained by scanning a clean sensor chip. Alternatively, since the fringes are constant across the scanning direction, a 'self-reference' image can be generated by taking a line cut from the raw image and repeating it in the scanning direction. The final image is then constructed by dividing the raw image by this self-reference image, which eliminates the need for an extra reference chip scan. After the removal of fringes, alignment marks (etched region) which appear as small bright spots are detected by applying a threshold to normalized red channel image, and the associated pixels in the green channel image are set to zero (Fig S-3). After this step only non-zero elements of the image are processed. Following the removal of fringes and alignment marks, the LUT is applied to the scaled image to assign each pixel to a thickness value, and the spots are then processed to find the height of the biological layer. The height of each spot is determined by subtracting the average thickness of an annulus around the spot from the average thickness of the spot's central area. Each spot's center is selected manually by the user. The central area is defined as the region inside a circle with a radius of 8 pixels (42.3 μm), and the annulus area is defined as the region between two circles with radii of 18 and 23 pixels (95.25 μm and 121.7 μm) as shown in Figure 2. As indicated previously only non-zero valued pixels are analyzed.

Sample Preparation. Silicon substrates purchased from Silicon Valley Microelectronics with thermal oxide of 100 nm are cut into 10 mm x 10 mm squares and cleaned by sonicating in acetone, rinsed with methanol and deionized (DI) water, and later dried with nitrogen. The surface functionalization technique used is explained elsewhere.²⁶ Briefly, clean SiO₂-Si chips are pretreated with 1 M NaOH for 30 min. After rinsing with DI water, chips are immersed in a polymeric solution (DMA-NAS-MAPS at 1% w/v concentration) (Lucidant Polymers) for 30 min, washed again with DI water, dried with nitrogen gas, and finally baked in the 80°C oven for 15 min.

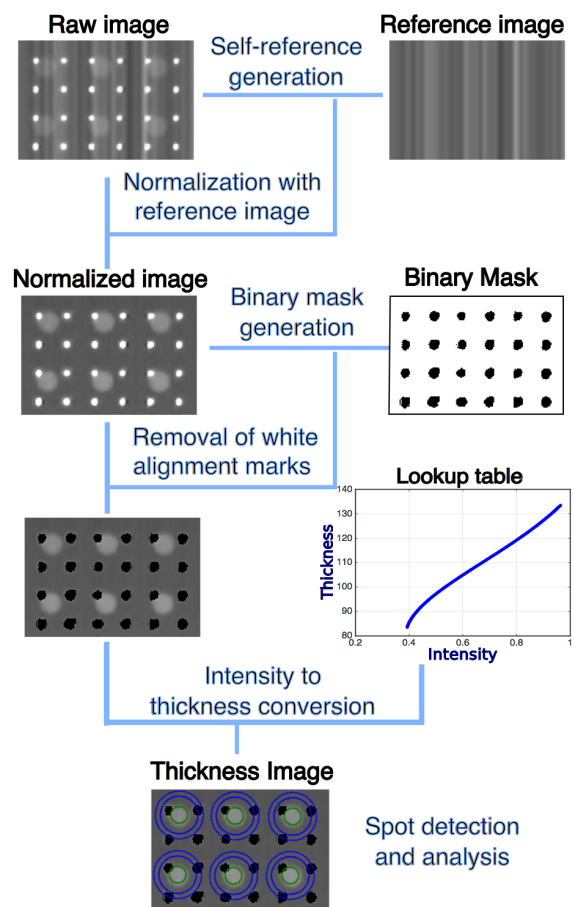


Figure 2 Data acquisition and processing. In raw images, scanning artifacts are observed, which are attributed to GRIN lens array. To get rid of these artifacts, the raw image is normalized using a self-generated reference image. After normalization, bright spots associated to etched regions on the sensor surface (alignment marks for spotting) are removed from the image by applying a threshold. A thickness value is then assigned to each pixel by using a lookup table generated previously with a calibration experiment. On this final image, spot centers are manually selected by the user and spot heights are calculated by subtracting the average thickness of a reference region outside the spot (area between blue circles) from the average thickness of the spot's central region (area inside the green circle).

The chips are stored in the desiccator until the microarray spotting is performed.

The sequence information of all the oligonucleotides (Integrated DNA Technologies) used in the experiments are shown in Table 1. DNA sequences were designed by using Oligo-Analyzer tool (Integrated DNA Technologies) to minimize the hairpin, self-dimer and heterodimer structures in order to increase the hybridization efficiency and prevent cross-hybridization. For DNA directed antibody immobilization, 40 mer probes were selected for increased elevation of the antibodies.²⁷ For DNA-DNA hybridization (A-A' hybridization), 40 bp length was selected randomly. 5 bp polyA sequence was added as a spacer to the antibody-linked DNA sequence (B'). All DNA sequences except A' were modified with amine groups at the 5' terminal. Surface probes are immobilized on the chip surface by binding to -NHS groups in the copolymer.

Table 1. Surface Probes (A, B and C) and their corresponding target sequences (A', B'-13F6 *).

Name	Sequence	Antibody	Info
A	5' ATC TGA ACC CAC CGC TAT TCC ATG CAC TTG ATT CCG AGG C 3'	-	Surface Probe
A'	5' GCC TCG GAA TCA AGT GCA TGG AAT AGC GGT GGG TTC AGA T 3'	-	Complementary to A
B	5' ATC CGA CCT TGA CAT CTC TAC CAC TGC GAC TAA CTC TGT A 3'	-	Surface Probe
B'-13F6	5' AAAAA TAC AGA GTT AGT CGC AGT GG 3'	13F6	Partially complementary to B, *Conjugated to 13F6 Ab
C	5' ACT TAG GAC TCA GTA CAG GAT AGA CTT CAG CGT GGT TGG A 3'	-	Unrelated sequence

B' sequence is conjugated to the antibody at the 5' terminal amine groups. The antibody against Ebola virus (EBOV) glycoprotein (13F6) used for DNA-directed antibody immobilization experiments is provided by Mapp Biopharmaceutical. 13F6 Antibody-DNA conjugates were synthesized by using Thunder-Link Oligo Conjugation Kit (Innova Biosciences).

DNA microarrays were created using a sciFLEXARRAYER S3 (Sciencion) piezoelectric arrayer. DNA molecules were spotted onto the functionalized sensor chip at a concentration of 50 μM in sodium phosphate buffer (150 mM, pH 8.5). After spotting, the chips were kept in a humid environment with 67% humidity overnight. Following the immobilization, they were first washed with 50 mM ethanolamine in 1x Tris-buffered saline (150 mM NaCl and 50 mM Tris-HCl, Fisher Scientific), pH=8.5, for 30 min, then washed with PBST (PBS with 0.1% Tween) for 30 min, rinsed with PBS and dried with nitrogen.

DNA microarrays were hybridized with target oligonucleotides at 1 μM concentration (to make sure that the depletion of the target is not limiting factor for hybridization) in PBS at room temperature for 1 hour. Following hybridization, microarrays were washed 3 times in 2x SSC for 3 min, once in 0.2x SSC for 3 min, and once in 0.1x SSC for 10 sec.

In DNA-directed antibody immobilization experiments, microarrays were hybridized with the 13F6-DNA conjugate at 10 $\mu\text{g}/\text{ml}$ in PBS at room temperature for 1 hour. After hybridization, chips were washed with PBS three times for 3 min, once with 0.5 M sodium nitrate buffer for 1 min, and once with cold 0.1 M sodium nitrate buffer for 10 seconds.²⁸

RESULTS AND DISCUSSION

Calibration Experiments. In order to calibrate our scanner against a standard IRIS instrument and generate a lookup table to convert pixel intensities to optical thickness, we used a chip with DNA microarray spots of varying height between 2 nm and 5 nm. Following the measurements using the IRIS, and generation of the look-up table, the chip is scanned with the scanner, intensity values are calibrated with IRIS, and the same look-up table is applied to the scanner image for conversion. IRIS and scanner images are shown in Fig. 3a, whereas Fig.3b shows the microarray spot heights calculated from the two images. This LUT is used for all following measurements with the scanner. In order to determine the noise floor, the same chip is measured 6 times in a one hour period. (10 frames averaged per measurement with 7 min intervals be-

tween measurements). The standard deviation of the height measurements of the same spot on the chip is found as 0.04 nm which corresponds to a biomass of 50 pg/mm^2 for proteins and 33 pg/mm^2 for DNA microarrays.²⁹ In commercial scanners, optical resolution is reported in dot per inch (dpi). However, this specification is mainly associated with the final interpolated image. In order to find the optical resolution of the modified scanner we implemented the ISO 12233 slanted edge method³⁰ and found the spatial resolution to be 12.6 μm (Fig. S-4).

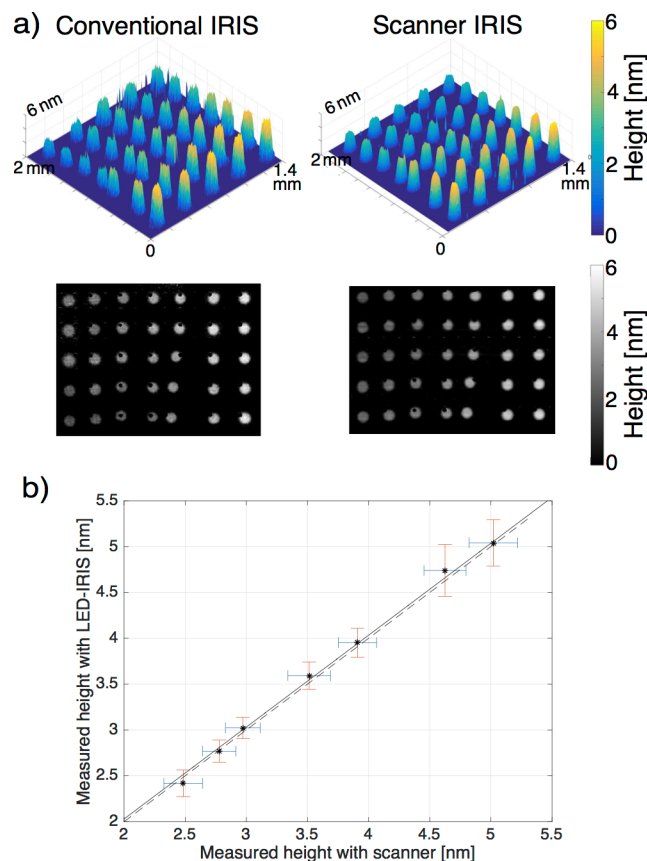


Figure 3 (a) Representing surface plots (top) and images (bottom) of the same chip with varying biomass, captured with conventional IRIS (left) and scanned with modified scanner (right). (b) Comparison of two setups. The dotted line represents equity, and line represents linear least-square regression.

DNA Hybridization Experiments. To create a DNA microarray with varying DNA surface densities, we spotted A sequence by mixing it with unrelated C sequence at different ratios (0%, 10%, 20%, 40%, 60%, 80%, 100%). We performed this mixing to keep the total spotted DNA concentration constant in order to provide uniform spot morphologies across different concentrations of A sequence. The complete array contains a total of 49 spots, 7 repeats from each mixing ratio. Following probe immobilization and washing steps, the chip is scanned to measure the initial DNA probe heights. Upon incubation with sample A', which is complementary to the A probe and not expected to hybridize to C, the chip scanned to measure the final spot heights. Pre-incubation and post-incubation images are visualized in greyscale as shown in Fig. 4a. The average initial and final heights of each replicated spot is shown in Fig. 4c. Error bars correspond to the standard deviation of the measured heights of the replicate spots. The thickness change due to the hybridization of the probe DNA, and the dependence of the height increase on the % mixing ratio of A and C sequences is observed.

DNA Directed Antibody Immobilization. The high spot-to-spot and chip-to-chip variation in protein microarrays in comparison to DNA microarrays is a bottleneck in their clinical use.^{6,31} For better spot morphologies and increased reproducibility of protein microarrays, immobilization through oligonucleotides have been suggested.^{32,33} This technique, DNA-directed antibody immobilization, has been shown to elevate the antibodies from the surface and improve their capture efficiencies.²⁷ To show the suitability of the scanner as a quality control instrument for antibody microarrays, we

conducted a DNA-directed antibody immobilization experiment (Fig. 4b).

DNA probes of two different sequences (B and C) mixed in varying ratios are spotted on the functionalized sensor surface, and the array is incubated with the B'-13F6 conjugate. Pre-incubation and post-incubation images are shown in Fig. 4b, where the quality of each individual spot can be inspected. The measured spot heights and spot-to-spot variations are indicated in Fig. 4c. As expected, since the size of the antibodies is larger in comparison to the size of the oligonucleotides, the antibody-conjugated DNA hybridization is detectable at a lower mixing ratio (10%) than DNA hybridization (shown in Fig. 4a, 20%). For the same reason, saturation of the spots is also observed for high mixing ratios (>80%).

CONCLUSIONS

In summary, we report the adaptation of a commercial flat-bed scanner to function as a label-free and high-throughput DNA and protein microarray imager through easy-to-implement hardware and software modifications. We provide a comparison of the system against a well-established label-free interferometric sensor (IRIS), and report a noise floor of 40 pm, which corresponds biomass of 50 pg/mm² and 33 pg/mm² for protein and DNA microarrays, respectively. With a cost as low as 100 USD, this platform can be used in label-free diagnostics applications, especially in low-resource settings. More importantly, it can easily be integrated into the existing protocols of fluorescence based detection assays to address variations across and between arrays by providing true quantitative quality control, as well as possible calibration of arrays of molecular probes (DNA or protein).

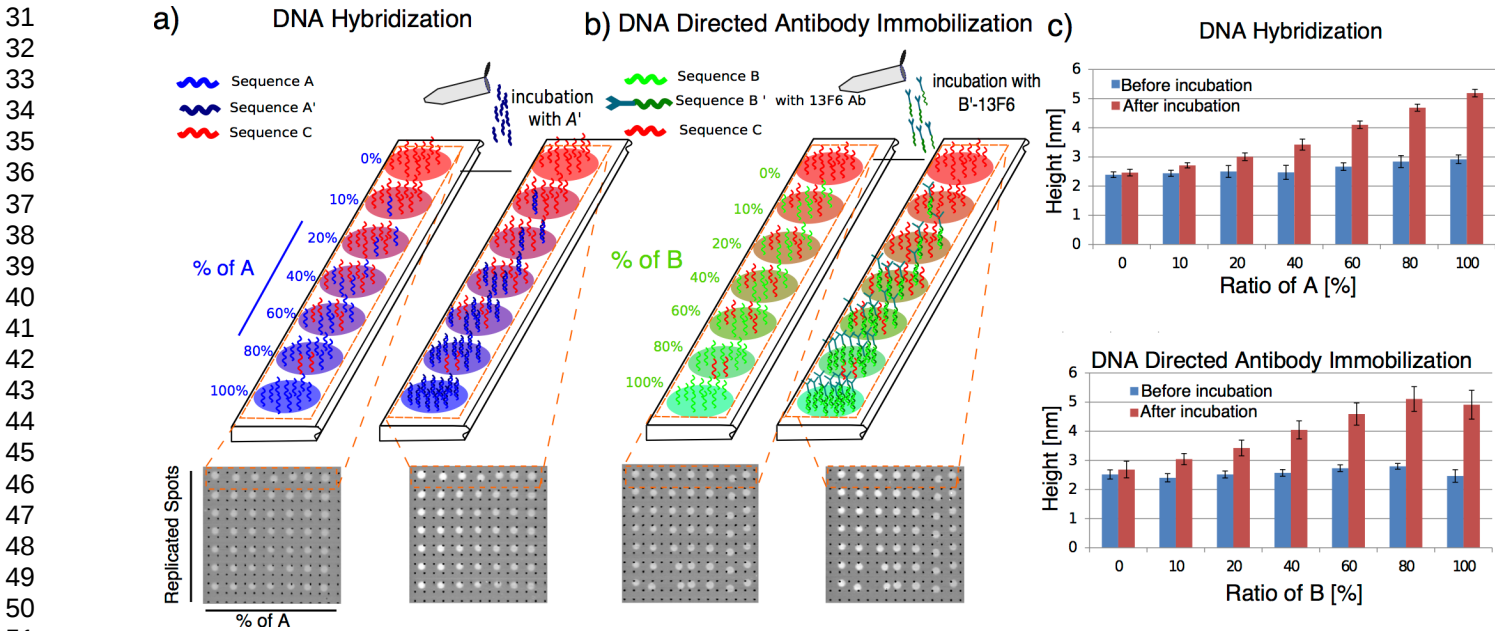


Figure 4: (a) DNA Hybridization Experiment. DNA spots are prepared by mixing A and C probes and then incubated with A' which is complementary to A only. Sensor chip is scanned both prior and after incubation (bottom). (b) DNA directed antibody immobilization experiment. DNA spots are prepared by mixing B and C probes and then incubated with B' -13F6 conjugate. (c) Average height of spots before and after hybridization for DNA hybridization experiment (top) and DNA directed antibody immobilization (bottom). Error bars represent 1 standard deviation of measured height among replicated spots.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Spatial resolution and depth of field measurements of the proposed biosensing platform. Details of the mechanical modifications, data processing and LUT generation (PDF).

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Notes

Authors declare no competing financial interest.

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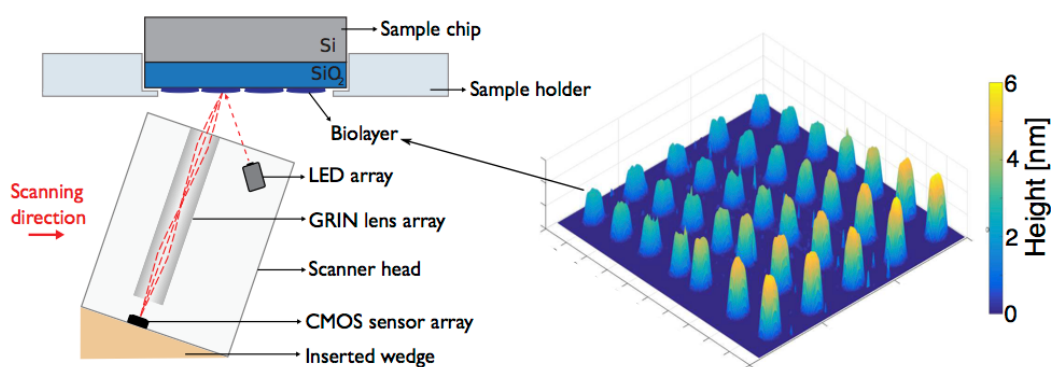
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for TOC only

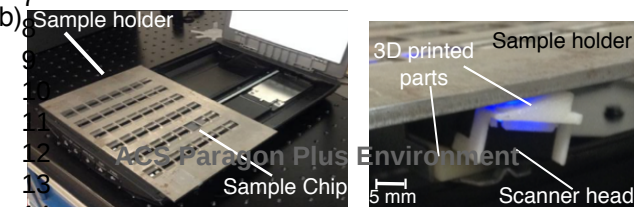
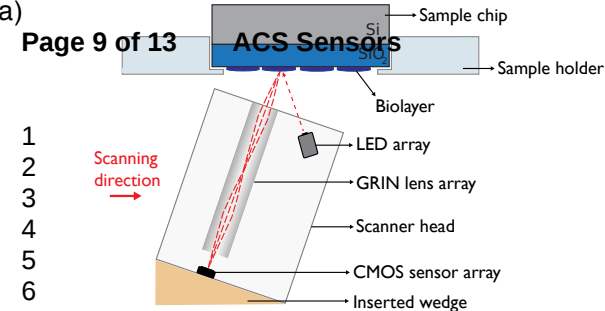


Figure 1

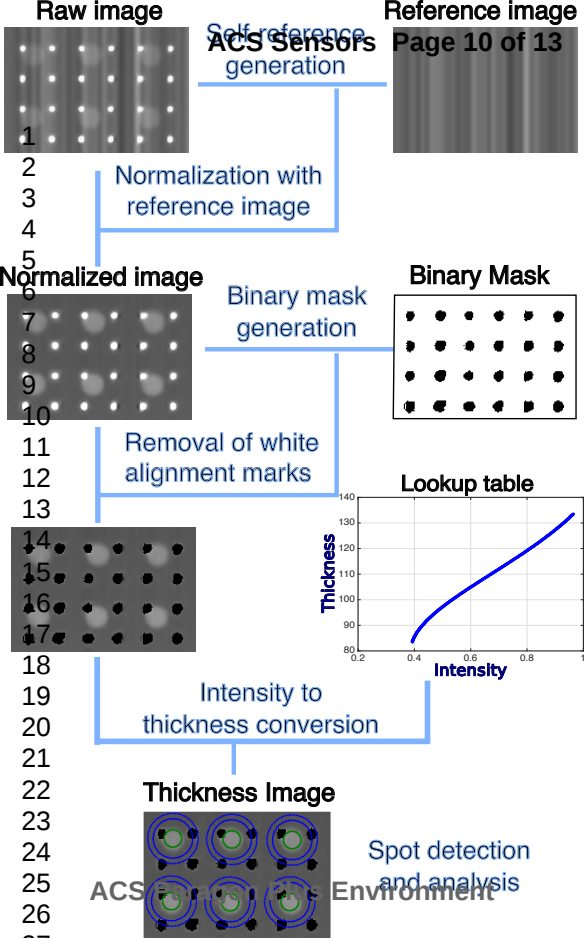


Figure 2

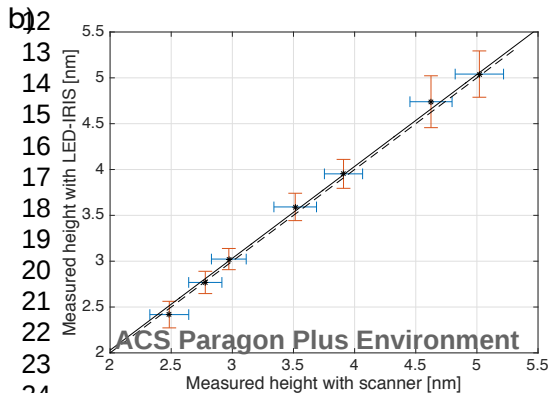
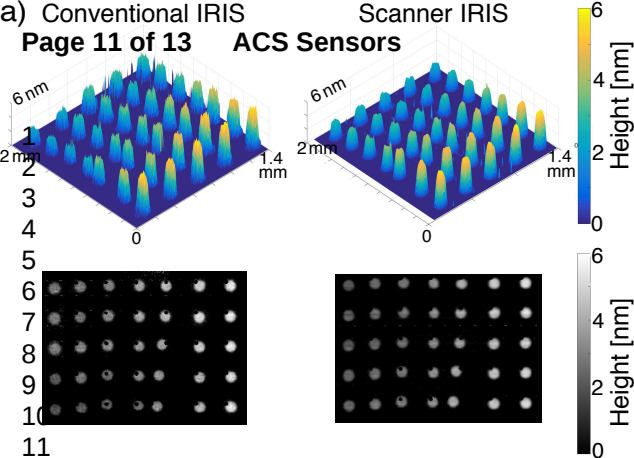


Figure 3

