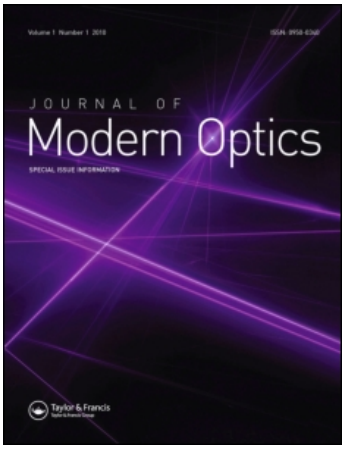

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SHORT COMMUNICATION

Self-referencing substrates for optical interferometric biosensors

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Optical interference is a powerful technique for monitoring surface topography or refractive index changes in a thin film layer. Reflectance spectroscopy provides label-free biosensing capability by monitoring small variations in interference signature resulting from optical path length changes from surface-adsorbed biomolecules. Spectral reflectance data can be acquired either by broad wavelength illumination and spectroscopy at a single point, thus necessitating scanning, or by varying the wavelength of illumination and imaging the reflected intensity allowing for acquisition of a spectral image of a large field of view simultaneously. In imaging modalities, intensity fluctuations of the illuminating light source couple into the detected signal, increasing the noise in measured surface profiles. This article introduces a simple technique for eliminating the effects of illumination light power fluctuations by fabricating on-substrate self-reference regions to measure and normalize for the incident intensity, simplifying the overall platform for reflection or transmission-based imaging biosensors. Experimental results demonstrate that the sensitivity performance using self-referencing is equivalent or better than an optimized system with an external reference.

Keywords: interferometry; imaging; label-free biosensor; high-throughput; DNA/protein microarray; silicon fabrication

1. Introduction

Optical interference based biosensors such as the pico-scope [1], Reflectometric Interference Spectroscopy (RIfS) [2], Molecular Interferometric Imaging (MI2) [3], Resonant Cavity Imaging Biosensor (RCIB) [4], spectral domain optical coherence phase microscopy [5], and Spectral Reflectance Imaging Biosensor (SRIB) [6] all invoke interference of light reflections from multiple interfaces on a substrate to detect accumulation (binding) on a surface. These systems have key applications in biomedical research and medical diagnostics as label-free sensing platforms for DNA and protein microarrays [7]. In all of these sensing modalities, the interference signatures are imaged as intensity changes across a surface allowing for optical path length changes corresponding to biomass accumulation to be detected at multiple locations simultaneously. The incident light undergoes multiple reflections owing to the structure of the layers and thus the reflected light acquires the corresponding spectral signature. In order to accurately characterize this signature it is necessary to monitor the intensity of the illumination source. Light sources, whether it is laser, lamp, LED, or other, are subject to fluctuations in light intensity. Causes for intensity fluctuations of the source include temperature changes, electric power fluctuations, and mechanical vibrations. These fluctuations lead to additional uncertainty in the recorded signal, reducing the measurement accuracy [8]. Thus, it is necessary, for high accuracy and high precision measurements, to compensate for these fluctuations in the illumination source.

Monitoring the illumination intensity with an external photodetector is perhaps the most common method to compensate for intensity fluctuations. The reference signal can be fed back as part of a closed loop control system to stabilize the illumination, or otherwise simply recorded for a post processing normalization [9]. While this external photodiode provides the necessary reference, its implementation requires additional optical components and splitting the illumination path and thus it is prone to misalignment and vibrations in opto-mechanical systems.

We propose and have implemented an alternative method that centers on the use of reference regions on the sample itself to compensate for incident light fluctuation. As all of the modalities listed above involve the processing of intensity images of the

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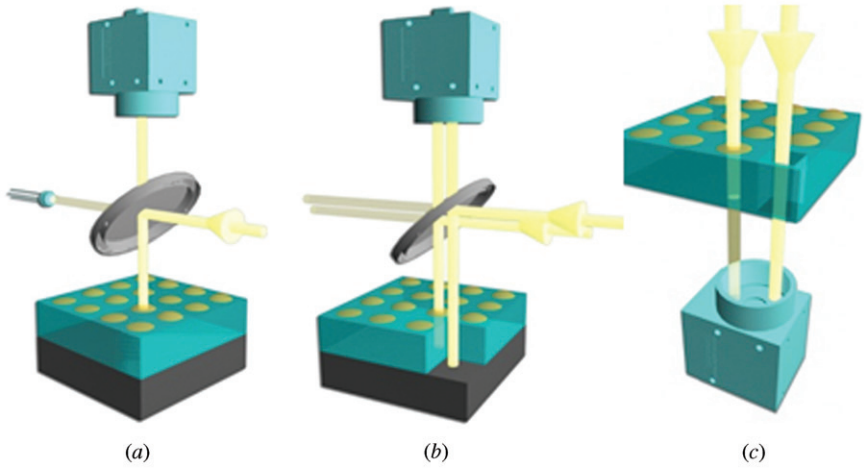

Figure 1. Implementation of self-referencing. Illustration comparing (*a*) traditional photodetector monitoring of incident light intensity, and the alternative self-referenced monitoring shown for both (*b*) reflection (IRIS with etched substrate) and (*c*) transmission techniques (substrate with thru-hole). Yellow circles indicate biomolecular probes. Two light beams are drawn in (*b*) and (*c*) simply for ease of representation. In actuality, there is clearly a single beam of incident light, of which, parts of the beam interact differently with the different regions of the sample. (The color version of this figure is included in the online version of the journal.)

sample to detect optical path changes, reference regions are essentially dedicated pixels on the images that correspond to reference regions of the sample. Data from these self-referencing regions can thus be acquired as part of the spectral image of the sample and be used to normalize the spectral information acquired from the rest of the field of view (FOV) to correct for temporal incident light fluctuations. Ideally, the reference regions are areas of the sample with a predictable reflection that is independent of the wavelength of illumination or any other variable under test. Considering that most materials are dispersive, a deterministic reflectance spectrum that does not vary during the sensing experiment can be utilized instead, i.e. the reflection spectrum of the reference regions should not change with biomolecular adsorption.

For reflection-based imaging systems, a spectrally invariant metal spot, for instance, can be placed on the sample within the imaging FOV to serve as a self-referencing region. We demonstrate the proof of principle with SRIB (recently renamed to the Interferometric Reflectance Imaging Sensor and will henceforth be called IRIS) in which reflection spectra on a SiO_2/Si structure yields information about the surface accumulation of biomolecules. We utilize the Si–air (or Si–solution) interface as the self-referencing region with deterministic reflectance spectra. These self-referencing regions are created by simply etching the oxide layer down to the Si surface in certain areas of the sample (discussed further). Likewise, for transmission-based imaging systems, the reference region can simply be a through hole in the sample that allows incident light to pass through without modification by the sample. Figure 1 illustrates the traditional photodetector method of intensity fluctuation monitoring that was used for IRIS (Figure 1(*a*)) as well as the concept of self-referencing for both IRIS, a reflection-based system (Figure 1(*b*)), and a typical transmission-based system (Figure 1(*c*)).

2. Materials and methods

The Interferometric Reflectance Imaging Sensor (IRIS) was used for validation of this self-referencing method. The IRIS is a high throughput, label-free optical biosensing platform [6]. It provides a means to screen many different interactions (protein–protein, DNA–protein, and DNA–DNA) simultaneously, on a single microarray. The IRIS sample consists of a silicon substrate with a thermally grown SiO_2 layer. The interference of light reflected from SiO_2 surface and Si–SiO_2 interface is monitored by illuminating the substrate with a tunable laser and capturing the reflected intensity with a CCD camera and thus acquiring a spectral image of the entire surface [9].

2.1. *Reference region fabrication and microarray spotting*

Reference regions were fabricated by patterning a 17 μm thick SiO_2 surface with S1813 photoresist and acid etching the patterns with BOE (6 : 1) to expose regions of bare Si substrate. Prior to spotting, the

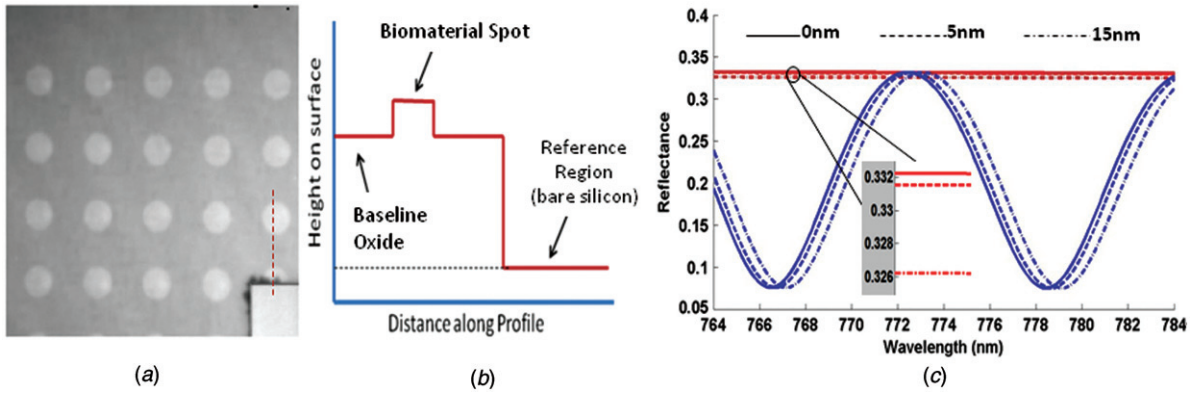

Figure 2. Sample and self-reference regions. (*a*) Post-processed image of HSA spotted sample showing self-reference region in bottom right corner. Dashed line shows the line profile depicted in the illustration in (*b*) of the height on the surface with respect to the distance along the profile drawn. (*c*) Simulated reflectance data for the oxide surface (blue) and bare Si reference region (red) over the IRIS sampling wavelength range with 0, 5, and 15 nm of binding. (The color version of this figure is included in the online version of the journal.)

surface was functionalized for covalent attachment of proteins [10,11]. To demonstrate the proof-of-concept, a simple protein array was imaged by the IRIS utilizing self-referencing. $0.5\,\mathrm{mg\,ml^{-1}}$ human serum albumin (HSA) was spotted on a substrate with reference regions. For the dynamic protein detection experiment, mouse IgG, rabbit IgG, and BSA were spotted at $0.5\,\mathrm{mg\,ml^{-1}}$ each. After overnight incubation in a humidity chamber, these substrates were washed and dried under argon.

2.2. *Si reference region theory*

A grayscale image of the HSA array sample taken by the IRIS is shown below in Figure 2(*a*) and is described schematically in Figure 2(*b*). The bare Si reference region is indicated by the bright square in the bottom right corner of the image. These regions are not functionalized and the only biomass accumulation would be due to non-specific binding. In typical experiments, non-specific binding is very small corresponding to a negligible dielectric film on the bare Si surface. Figure 2(*c*) shows simulated reflectance spectra for various accumulations of binding (0, 5, and 15 nm – where 1 nm thickness corresponds to approximately to $1\,\mathrm{ng\,mm^{-2}}$ protein mass) on the oxide surface and the bare Si reference region. The reflectance curves have negligible wavelength dependence for the bare Si over the sampled wavelength range even when substantial non-specific binding is considered. Only a wavelength independent reduction in reflected signal is observed in contrast to the significant shift in the spectral signature with similar biomass accumulation on the detection (oxide) surface. Only the relative values of the intensity at different wavelengths are relevant in the analysis of the IRIS signal and thus utilizing the self-reference data to compensate for intensity fluctuations yields identical results to that obtained from an ideal reference. Thus, the reference regions represent an effective method for compensating for intensity fluctuations even in the presence of comparable biomass accumulation on them due to non-specific binding.

2.3. *Experimental procedure*

To assess self-referencing regions in providing sensitive, repeatable measurements, 19 consecutive test measurements were acquired of the same field of view without moving the HSA sample. For each image, the signals were recorded and the standard deviations of the observed spot heights were calculated. For performance comparison, a calibrated photodetector was used to measure the incident light intensity at each wavelength of light during data acquisition as performed [9]. The optical path difference (OPD) on the spots were calculated from the acquired data using multiple analysis methods: post-processing using the reference region for compensation, post-processing using the external photodetector reference, and post-processing of raw data that is not corrected with either method, thus prone to intensity fluctuations. On a measurement-to-measurement basis, these post-processing schemes attempt to effectively eliminate the error induced by slightly varying laser intensities. After post-processing, the average signal for 15 protein spots was found for each measurement by subtracting the average height of the background from the average height within the spot. The process was completed for each of the 19 scans and the standard deviation in the

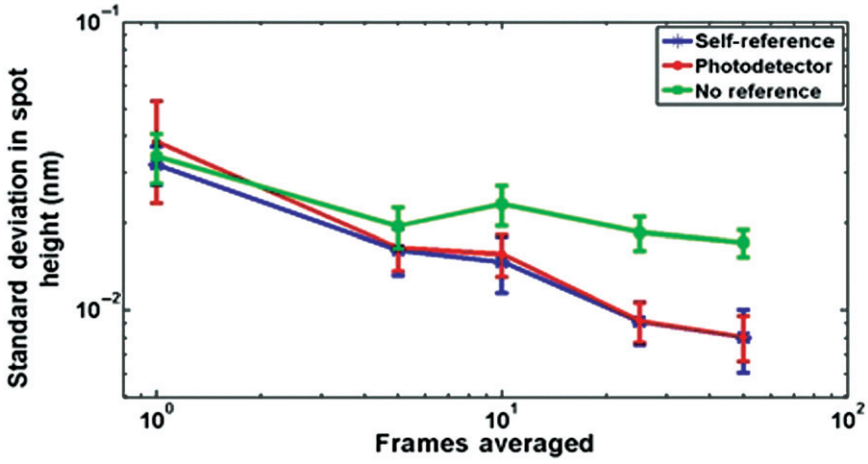

Figure 3. Sensitivity results. Standard deviation in measured spot height over 19 consecutive measurements averaged for 15 spots using self-referencing (blue) and the photodiode (red) for normalization as well as no referencing at all (green) over range of frames averaged. (The color version of this figure is included in the online version of the journal.)

measured spot height was found. This measure of temporal noise due to laser intensity fluctuations is an important measure of stability and sensitivity for intended applications such as real-time binding analysis.

The dynamic protein detection experiment was performed using the three immobilized probes described in Section 2.2. The sample was secured in a custom flow cell with a 500 µl volume and IRIS measurements were taken continuously as solutions were driven through the chamber at 400 µl min^{-1} using a peristaltic pump. 1×PBS was first flowed into the chamber for 15 min. Next, anti-mouse IgG at 10 µg ml^{-1} was flowed for 15 min, followed by 1×PBS again for 15 min, and finally, anti-rabbit IgG at 10 µg ml^{-1} for 15 min. 50 frames were averaged for these measurements, and once again, the OPD on each spot was calculated using the data analysis and three post-processing schemes described previously.

3. Results and discussion

Figure 3 shows the standard deviation in measured spot heights over 19 measurements for 15 spots in the 1.5 mm × 1.5 mm field of view shown in Figure 2. The height of each spot in the set of 19 measurements was determined using the three modes of data processing discussed in the previous section. During IRIS data acquisition, images are averaged at every wavelength to increase the signal-to-noise ratio. Figure 3 shows the resulting standard deviations on the signal using the three methods for different numbers of averaged frames. The standard deviation of measured spot height is 32 pm when no averaging is applied whereas the accuracy improves to 8 pm when 50 frames are averaged for every wavelength during data acquisition.

This experiment shows that the on-substrate referencing method of normalizing for fluctuations in incident light intensity is effective and on par with the standard method of photodetector post-processing. Normalization of light intensity during data processing leads to approximately a two-fold improvement in the spot height standard deviation over the 19 scans (Figure 3) from 17 to 8 pm at 50 frames averaged. The self-referencing technique yields an identical standard deviation as photodetector normalization, showing that the new self-referencing technique is an effective replacement that simplifies the overall optical system.

The dynamic binding experiment demonstrates the utility of self-referencing for temporal measurements of specific binding interactions in a high-throughput, multi-analyte assay. Figure 4(*a*) shows a small region of the array used in this experiment. Figure 4(*b*) shows the dynamic binding curves for the same data using the three modes of post-processing described. The curves remain flat for all conditions until the 15 min point when anti-mouse IgG is flowed and specific binding of 0.8 nm to mouse IgG is observed (red-solid lines) by the 30 min time point. The curves are flat again during the PBS flow from minutes 31–45. During the 46–60 min time window, specific binding to 1 nm of height increase is observed on the rabbit IgG spots (blue-dashed lines) while anti-rabbit IgG was flowed. The BSA spots show minor non-specific binding (0.1 nm) and there is some non-specific binding of anti-mouse IgG to rabbit IgG spots from 16–30 min due to some cross-reactivity. It is clear just from looking at the three plots that photodetector referencing decreases temporal fluctuations

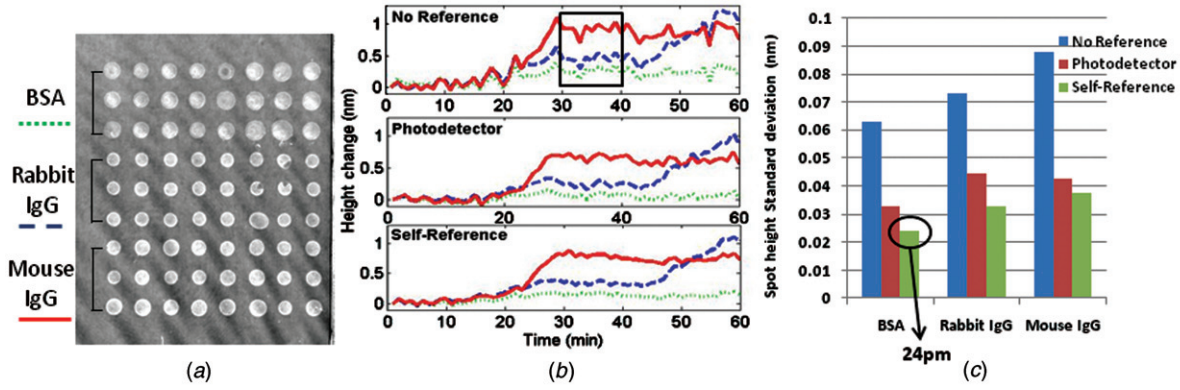

Figure 4. Dynamic binding results. (*a*) Small region of post-processed image of dynamic protein detection sample showing the three conditions of immobilized probes. (*b*) Plots showing height increase over time for BSA (green-dotted), rabbit IgG (blue-dashed), and mouse IgG (red-solid) as complementary analytes were flowed. Results from the three types of post-processing are shown. (*c*) Standard deviation in spot height for each immobilized probe using the three types of post-processing from minutes 31–40 of the experiment. (The color version of this figure is included in the online version of the journal.)

compared to no referencing, and self-referencing actually improves this noise even more so. A closer look at the stable 31–40 min time window in each post-processing plot shows that standard deviation in measured spot height is lowest when using self-referencing, 24 pm for BSA compared to 33 pm for BSA using the photodetector, and this is consistently true for each condition (Figure 4(*c*)). While this method is shown with three different immobilized probes of 24 spots, self-referencing is easily scalable to more high-throughput arrays.

While the HSA experiment showed that at 50 frames averaged self-referencing yielded similar noise results as photodetector referencing, we believe that the self-referencing method yielded better noise results in the dynamic binding experiment because it was conducted in solution. When making dry measurements, as was the case in the HSA experiment, the photodetector and self-reference regions both directly monitor the light source, with only static optics intervening in the optical paths. However, in solution based experiments, the temporal effects of a dynamic buffer solution on incident light intensity on the sample are also taken into account by self-referencing, while it is not when using the photodetector. Therefore, we believe that for wet measurements, self-referencing is a better monitor of incident light on the sample than a photodetector.

4. Conclusions

Utilizing on-substrate referencing in lieu of an external photodetector, simplifies the optical system by eliminating the need to divide the light path. This improvement can assist in making systems more compact and field-usable as fewer opto-mechanical and electronic components will be needed in these systems, and added alignment and stability requirements can be avoided. Aside from additional components, post-processing the primary measured data from the camera alone is likely to be less error prone than splitting the beam path where wavelength or polarization dependencies and temporal variations can be problematic. In our experimental comparison, the external reference photodiode has been carefully aligned and experiments have been carried out on a vibration isolation table.

The comparable, or better, sensitivity shown by on-substrate referencing on the IRIS validates this technique as an effective practice for use in optical interference biosensor modalities. The concept is simple and general, such that it is applicable not only to reflection-based imaging modalities, but also transmission-based imaging modalities. For example, transmission techniques such as RIfS would employ a through hole in the substrate to serve as a reference region (Figure 1) serving as an incident light intensity monitor. This on-substrate self-referencing, with its simple fabrication protocol and implementation, can be easily applied to a variety of imaging biosensors to enhance their precision and accuracy, while simplifying the overall platform.

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References

[1] Nikitin, P.I.; Gorshkov, B.G. *Sens. Actuators B* **2005,** *111–112,* 500–504.

[2] Gauglitz, G. *Rev. Sci. Instrum.* **2005,** *76,* 062224.
[3] Zhao, M.; Wang, X.; Nolte, D. *Opt. Express* **2008,** *16,* 7102–7118.
[4] Bergstein, D.A.; Özkumur, E.; Wu, A.C.; Yalcin, A.; Colson, J.R.; Needham, J.W.; Irani, R.J.; Gershoni, J.M.; Goldberg, B.B.; DeLisi, D.; Ruane, M.R.; Ünlü, M.S. *IEEE J. Sel. Top. Quantum Electron.* **2008,** *14,* 131–139.
[5] Joo, C.; Özkumur, E.; Ünlü, M.S.; de Boer, J.F. *Biosens. Bioelectron.* **2009,** *25,* 275–281.
[6] Özkumur, E.; Needham, J.W.; Bergstein, D.A.; Gonzalez, R.; Cabodi, M.; Gershoni, J.; Goldberg, B.B.; Ünlü, M.S. *Proc. Natl. Acad. Sci. U.S.A.* **2008,** *105,* 7988–7992.
[7] Stears, R.L.; Martinsky, T.; Schena, M. *Nat. Med.* **2003,** *9,* 140–145.
[8] Wang, X.; Lu, H.; Wang, X.; Yu, D.; Qian, F.; Fang, Z. *J. Opt. A: Pure Appl. Opt.* **2001,** *3,* 222–224.
[9] Özkumur, E.; Yalcin, A.; Cretich, M.; Lopez, C.A.; Bergstein, D.A.; Goldberg, B.B.; Chiari, M.; Ünlü, M.S. *Biosens. Bioelectron.* **2009,** *25,* 167–72.
[10] Cretich, M.; Pirri, G.; Damin, F.; Solinas, I.; Chiari, M. *Anal. Biochem.* **2004,** *332,* 67–74.
[11] Yalcin, A.; Damin, F.; Özkumur, E.; Di Carlo, G.; Goldberg, B.B.; Chiari, M.; Ünlü, M.S. *Anal. Chem.* **2009,** *81,* 625–630.