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Using Photonic Crystal Enhanced Fluorescence on Quartz Substrates to Improve the Sensitivity of DNA Microarrays

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Abstract— **Gene expression analysis of low abundance genes remains difficult when DNA microarrays are performed on standard glass substrates. However, we have shown that by using photonic crystals (PC) made on quartz substrates, the fluorescence intensity of Cyanine-5 (Cy5) labeled microarray spots is greatly enhanced. In a 1-color microarray experiment studying gene expression of soybean cotyledon tissue, an average signal enhancement factor of 17.8x was observed on the PC. Furthermore, twice as many genes were detectable on these PCs as compared to glass. By improving the sensitivity of this fluorescent assay, low expression genes that were undetectable on glass were quantified on the PC.**

I. INTRODUCTION

While DNA microarrays are a platform for the highly parallel study of expression of several genes of interest, usually the fluorescence intensity of only high expression genes - a small fraction of all genes in a cell population - can be detected above the noise in the experiment. However, the profiling of low expression genes, many of which have important housekeeping functions, is of keen biological interest. To better quantify low abundance genes, nano-patterned structures that intensify the electric field intensity surrounding surface-bound fluorophores, and in turn, enhance the fluorescence from common microarray dyes have been pursued [1]. But, unlike surface PCs, such structures are not fabricated using methods that can produce surfaces that are uniform over the areas necessary for microarrays comprised of thousands of capture probes, or in the format of standard microscope slides. Furthermore, since substrate auto-fluorescence is an important contributor to the experimental noise in a microarray, the choice of this material is critical. By addressing these three important fabrication considerations, we show that PCs made on low fluorescence quartz substrates can be integrated easily with traditional microarray protocols to significantly increase the dynamic range of such experiments.

The PC used here is composed of a one-dimensional periodic structure formed on a low refractive index quartz surface that is coated with a high refractive index layer of TiO_2. This periodic modulation in refractive index along the device surface gives rise to optical resonances when the device is illuminated at a particular wavelength and incident angle combination. When optical resonances are spectrally overlapped with the excitation and emission wavelengths of a fluorophore, they enhance the optical fields near the surface of the device and also spatially bias fluorescence emission for optimal collection. These two resonances, working through two distinct mechanisms, are combined to produce a multiplicative fluorescence signal enhancement [2]. This paper reports the application of quartz PCs to a 1-color DNA microarray that profiles genes expression in cotyledon tissue from soybeans. Three PCs with good optical uniformity over the area of microscope slides, fabricated using nano-imprint lithography, were paired with commercial microarray glass slides and studied under identical experimental conditions. Strong fluorescence enhancement on the PC resulted in substantial enhancements in spot intensity and the total number of genes detected on the PC as compared to glass. These results demonstrate significant gains in the sensitivity of microarray experiments.

II. MATERIALS AND METHODS

Device Fabrication and Characterization

The geometric parameters of the quartz PCs were first determined using Rigorous Coupled-Wave Analysis simulations (DiffractMOD, RSoft Design Group, Inc.) such that the device resonant modes were aligned to the excitation and emission wavelengths of the Cy5 fluorophore. Specifically, these PCs have a transverse magnetic (TM) polarized (corresponding to an electric field vector oriented perpendicular to the grating) resonant mode at the Cy5 excitation peak $\lambda_{excitation}$ = 632.8 nm that amplifies fluorophore excitation by enhancing the near-fields [3]. Furthermore, these devices have a second TM mode close to $\lambda_{emission}$ = 690 nm that lies in the band of emission wavelengths for Cy-5. This second TM mode applies a

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spatial bias to the fluorophore emissions to maximize light collection [4].

The quartz PCs were fabricated using a step-and-flash nanoimprint lithography technique as described previously [5]. Briefly, a template with the negative grating pattern was prepared over a 9 x 9 mm^2 area via electron beam lithography. Quartz wafers (4 in. in diameter) with ultra low auto-fluorescence were cleaned, planarized, and prepared for imprint by dispensing a uniform layer of an imprint resist. The imprint process was then performed (Imprio-55, Molecular Imprints, Inc.) where the template was slowly pressed against the resist and the resist was then cured by UV exposure. The template was released to reveal the transferred grating pattern on the resist layer and this step-and-flash process was repeated to cover the entire wafer surface. Finally, a set of precisely timed reactive ion etching steps were performed to transfer the imprinted pattern into the quartz substrate. The wafer was then cleaned and diced into standard microscope slides (1 x 3 in^2.) and fabrication was completed by sputtering a 160 nm thick layer of TiO_2 on the slides.

The surface characteristics of the finished PCs were profiled with an atomic force microscope (AFM) (Dimension 3000, Digital Instruments) to compare the device experimental dimensions to the initial design parameters. Next, the optical properties of the PCs mounted on an angle-adjustable stage was obtained by illuminating them with TM polarized, broadband light and collecting the transmitted light with a UV-visible light spectrometer (Ocean Optics). The transmission spectra of the two TM modes contributing to the fluorescence enhancement of Cy5 were obtained. The device resonant angle ($\theta_{excitation}$) for the off normal TM mode located at $\lambda_{excitation}$=632.8 nm was also determined.

Microarray Printing and Hybridization

The PC slides were functionalized with an epoxysilane-based surface chemistry described previously [6]. Commercially silanized glass slides (Corning GAPS II) were used as control slides in the microarray experiments. A previously reported [7] set of 192 oligonucleotides consisting of soybean genes were printed in replicates of 40, for a total of 7680 spots per slide. The 70-mer oligonucleotides were printed (QArray^2, Genetix) on three PCs matched with glass control slides. Printed slides were incubated overnight and then UV crosslinked. Cotyledon RNA was extracted from freeze dried soybeans seeds (*Glycine max* cultivar Williams). The total RNA, extracted from the separated cotyledons, was purified and labeled with Cy5 by reverse transcription. Printed slides were blocked with bovine serum albumin and then hybridized overnight at 42^{o}C. Approximately 40 µg of total RNA was used per slide. All slides received identical treatments throughout the assay.

Fluorescence Data Acquisition and Analysis

All slides were scanned using a confocal microarray scanner (LS Reloaded,Tecan) using a TM polarized laser (λ= 632.8nm) and an emission filter with a range of 670-710 nm. All slides were scanned at identical PMT gain settings and at a pixel resolution of 10 µm. Glass slides were scanned at normal incidence (0 degrees) while the PCs were scanned at their respective resonant angles ($\theta_{excitation}$). Spot segmentation and intensity calculations of the fluorescence scans were performed using Genepix Pro 6.1 (Molecular Devices). Spot SNR was calculated as the local background subtracted spot intensity divided by the standard deviation of the local background.

III. RESULTS

Surface and Optical Characteristics of the PCs

The schematic of the quartz PC design optimized to have two TM modes that enhance the fluorescence from Cy5 is shown in Fig. 1(a). The representative surface profile of the PC, obtained using an AFM showed good agreement in dimensions between the fabricated devices and this design (see Fig. 1(b)). A completed PC that has been diced to the dimensions of a microscope slide is presented in Fig. 1(c).

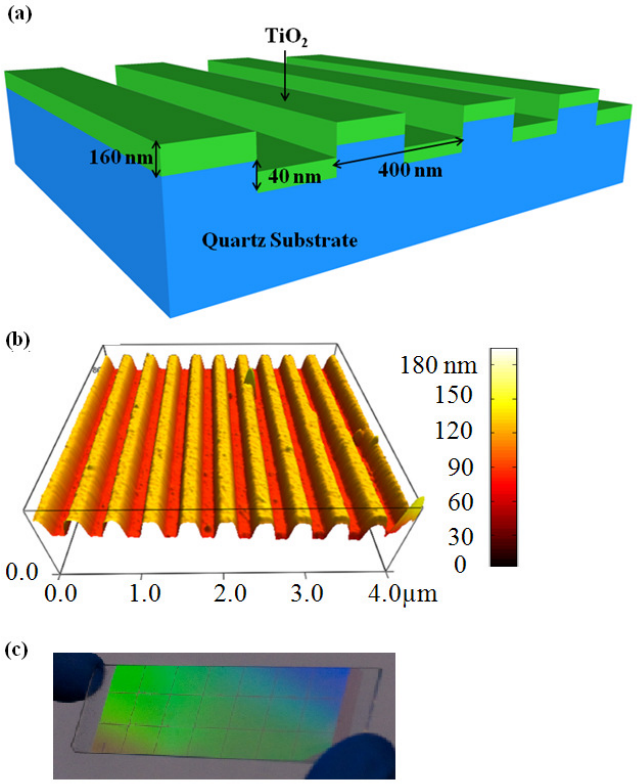

Fig. 1: (a) Schematic of the PC design and device dimensions. Grating period = 400 nm, duty cycle = 50%, grating depth = 40 nm, TiO2 thickness = 160 nm. (b) AFM surface profile of a completed PC (with TiO_2) with a measured grating period of 402 nm and a grating depth of 44 nm. (c) Image of a quartz PC diced to the dimensions of a microscope slide (1 x 3 in^2.)

A PC resonance can be excited by varying either the wavelength or the angle of the illumination that is incident on the device. The transmission spectra of a PC slide presented in Fig. 2 indicates the presence of two narrow PC resonances of interest obtained at two different illumination angles. At an illumination angle ($\theta_{excitation}$) of 9^o, there exists a resonance at the Cy5 excitation peak of 632.8 nm.

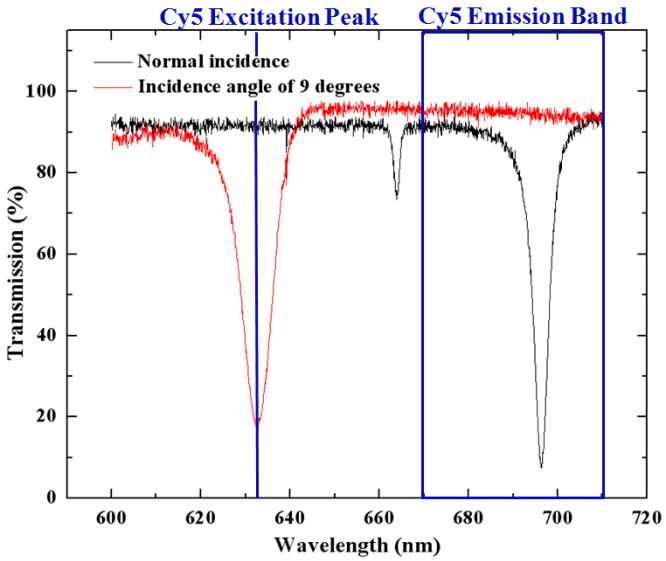

Fig. 2: Optical transmission measurements of a quartz PC when illuminated with TM polarized and collimated white light at two different incidence angles. When illuminated at an incidence angle of 9 degrees, there exists a resonant mode (red) that overlaps the Cy5 excitation wavelength of 632.8nm (blue line). For normal incidence illumination, there exists a resonant mode (black) that overlaps the Cy5 emission filter wavelength range of 670-710 nm (blue box).

A second resonance, centered at a wavelength of 696 nm, was obtained at normal incidence and overlaps the Cy5 emission filter band. Within any given slide, the observed maximum standard deviation in the resonant wavelength at normal incidence was 0.8 nm and was smaller than the spectral width of that resonance (full width at half maximum of 1.3nm). For large area microarrays, such high spectral uniformity throughout individual slides is critical to minimizing any position-biased fluorescence enhancement.

Microarray Spot Fluorescence Intensity Enhancements on PCs

Post-hybrization images of an identical sub-array on the PC and glass slide in Figures 3 (a,b) depict observed spot fluorescence intensity enhancement on the PC. Not only are signals from high expressing genes enhanced on the PC (Figure 3(c)), but several low expressors that cannot be differentiated from the background noise on the glass slide are detectable on the PC (Figure 3(d)). All 40 replicates of each gene were averaged for each slide and a ratio of the average background subtracted spot intensity on the PC to that on the paired glass was calculated for each gene. The average spot intensity enhancement was found to be 17.8x.

Enhancement in the Number of Genes detected on PCs

More importantly, the impact of spot intensity enhancements was observed in SNR enhancements and the increased number of genes that were detectable on the PC as compared to glass. In this analysis, a gene was defined as detectable if the replicate-averaged gene SNR was greater than 3. As shown in Figure 4, for a given PC-glass pair, only

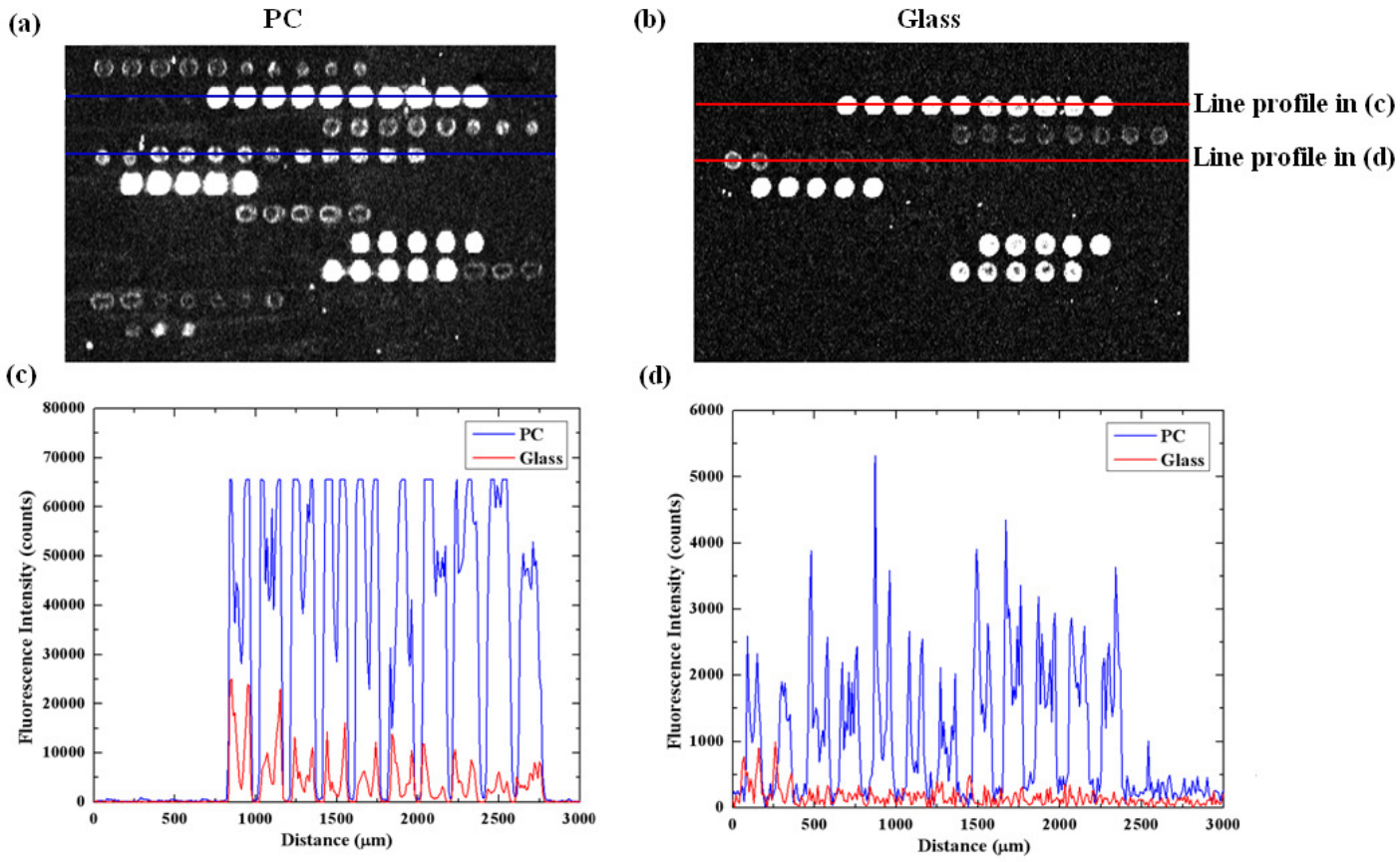

Fig. 3: Fluorescence images on a PC (a) and glass (b) of the same sub-array obtained at identical microarray gain settings. Contrast and brightness has been optimized on each image to maximize feature visability. Line profiles (c) and (d) show examples of signal enhancement of high and low expression genes, respectively.

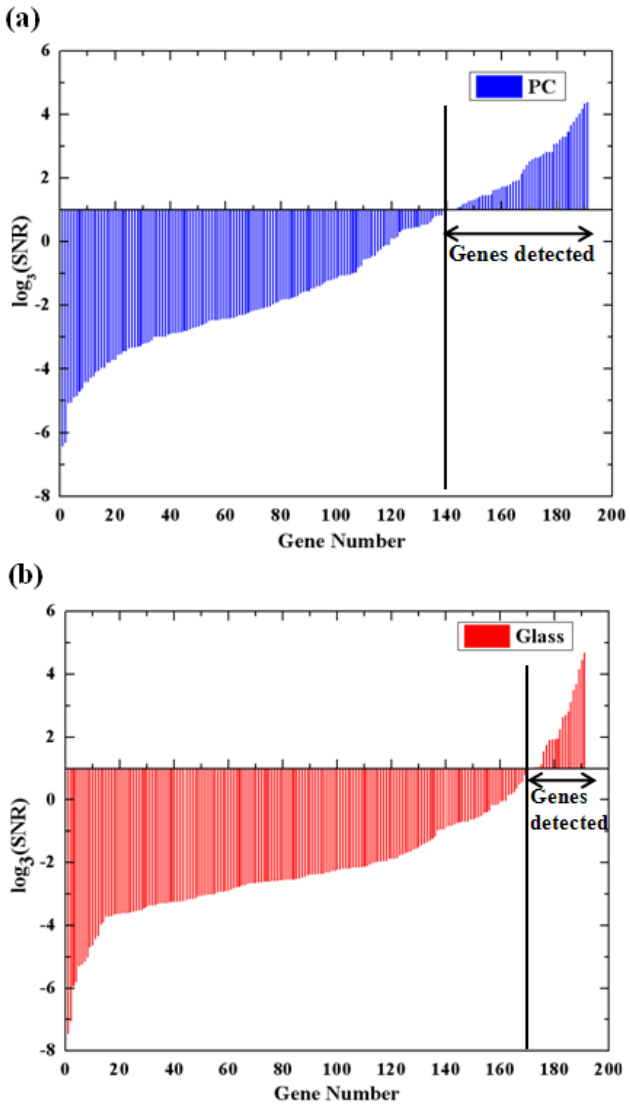

Fig. 4: The logarithm (base 3) values of replicate-averaged SNR for all 192 genes on the PC (a) and glass (b). Genes with SNR > 3 or correspondingly $\log_3(\text{SNR}) > 1$ are classified as detected. 51 genes were detected on the PC while only 26 were detected on the control glass slide.

13.5% of all genes were detectable on the glass slide while, 26.6% of all genes were detectable on the PC. In comparing all three PCs against their paired glass slides, an average of twice as many genes was detectable on the PC.

IV. DISCUSSION

Many of the highly expressed genes, detected both on the glass and PC, encode storage proteins which are abundant during this stage of seed development. The additional genes detected only on the PC represent enzymes and important regulatory transcription factors that are expressed at lower levels. Transcription factors provide genetic control over development and can also be important markers of disease state. The ability of the PC to detect these low abundance transcripts is reflected by the lower average fluorescence intensity of 515 counts for genes detected only on the PC as compared to an average fluorescence intensity of 3280 counts for genes detected on glass.

The quartz based PCs used in this work offer some unique advantages for DNA microarray applications over previously reported work using PCs based on plastic substrates with polymer gratings. Substrate auto fluorescence is an important source of noise in a microarray experiment, and increases when illuminated with higher energy (or lower wavelength) photons. The quartz PCs have a substrate fluorescence that is 15 times lower than plastic PCs. This is an important consideration when designing a PC to enhance fluorescence emission from Cyanine-3 (Cy-3, $\lambda_{\text{excitation}}$ = 536 nm), another fluorophore that is routinely used in DNA microarrays. Devices fabricated on low-fluorescence quartz substrates and designed for multiple fluorophore enhancements are being pursued for 2-color DNA microarray applications.

In conclusion, we have demonstrated that PC enhanced fluorescence using quartz substrates have significantly improved the sensitivity of a DNA microarray. Twice as many genes were detected over substrate noise on the PC compared to glass using a 1-color microarray.

V. REFERENCES

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