Application of Photonic Crystal Enhanced Fluorescence to Antibody Microarrays

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Abstract: A photonic crystal surface is utilized for detection of cancer biomarkers in a fluorescent-tagged protein microarray assay. The results indicates that the detection limit of assays are reduced by up to 90% through resonant illumination.

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1. Introduction

Antibody microarrays have potential applications as a clinical tool in disease diagnosis and drug discovery [1]. Through the use of calibration standards, protein microarrays provide measurements of analyte concentration that are highly quantitative [2], and sandwich assay protocols have been developed that demonstrate extremely low levels of nonspecific detection through the use of fluorophore-tagged secondary antibodies that enable multiplexed detection of cancer biomarkers in serum [3]. This paper extends our previous work [4] to the first demonstration of a photonic crystal enhanced fluorescence (PCEF) antibody microarray.

2. PC fabrication, immunoassay and data analysis

PCEF surfaces are designed to provide two distinct resonances [5]: TM-polarized resonance at the same wavelength as a laser that is used to excite a cyanine-5 (Cy5) dye at 633 nm, resulting in elevated electric field magnitude in an evanescent field region (enhanced excitation) and TE-polarized resonance at the Cy5 emission wavelength (690 nm), resulting in increased photon collection efficiency (enhanced extraction). The periodic surface structure used in this work was formed from a polymer material on a flexible plastic substrate using replica molding process [6]. The periodic surface structure was subsequently coated with SiO_2 and TiO_2, where a high refractive index of TiO_2 is necessary to establish the formation of guided mode resonance. The schematic of a cross section and scanning electron micrograph (SEM) of a top view of a PC are shown in Fig. 1a,b. Microscope-slide-sized rectangles (25x75 mm) of PC on a plastic substrate were attached to glass microscope slides with clear adhesive film. The device resonance condition was observed by measuring the dip in the transmission spectrum when the PC was subjected to broadband illumination at normal incidence, as shown in Fig. 1c.

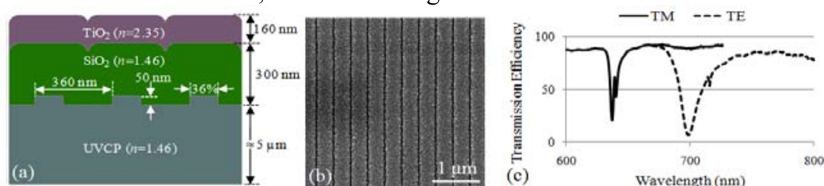

Fig. 1. (a) A schematic cross section of the PC design used in this work. The grating period =360 nm, grating depth=50 nm, SiO_2 thickness=300 nm, TiO_2 thickness=160 nm, and duty cycle=36%. (b) Top view of a SEM image of the grating structure. (c) Transmission spectrum of the photonic crystal at normal incidence. The resonance wavelength for the TM polarization (solid curve) is at λ=629 nm and the resonance for TE polarization (dashed curve) occurs around λ=690 nm.

A panel of 24 human breast cancer biomarkers [3] was selected to demonstrate the PCEF antibody microarray using a mixture of all 24 analytes. The dose-dependent response curves was generated using a seven-fold dilution series of the analyte mixture. A sandwich assay was used in which a cocktail of secondary (detection) antibodies are exposed to the array after analyte hybridization with first (capture) antobodies to eliminate nonspecific interaction between the assays, while Streptavidin-conjugated Cy5 was used to tag the secondary antibodies [3]. A confocal microarray scanner (LS Reloaded, Tecan) equipped with a λ=633 nm laser and user-adjustable incident angle was used to image the Cy5 fluorescent signal on the slide. ScanArray Express software (Perkin-Elmer) was used to quantify the spot intensities. ProMAT software, developed by Pacific Northwest National Laboratory specifically for

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the analysis of ELISA microarray data, was used to generate dose-response curves by fitting the fluorescence data to a four-parameter logistic curves [7]. The limit of detection (LOD) was also calculated by ProMAT as the concentration point on the standard curve corresponding to the mean plus three standard deviations of the log-transformed fluorescence intensities.

3. Results and discussion

The effects of PC enhanced fluorescence can be determined by comparing the fluorescence output under the following two conditions: (a) when the excitation laser incident angle is adjusted to illuminate the PC at the resonant angle ("on-resonance", ~0°), and (b) when the excitation angle of incidence is selected to not coincide with the resonant coupling condition ("off-resonance", 20°). The layout of the antibody for each replicate is shown in Fig. 2a where the erratic assay responses are highlighted in bold font. The failed assays result from missing spots during printing process or nonfunctional reagents due to denaturation during storage, which were excluded from further analysis. The fluorescent images (Fig. 2b,c) illustrate the observed enhanced fluorescence intensity where both images have the same photomultiplier tube gain settings. By scanning the PC at its resonant angle, the fluorescence intensity was enhanced by factors of 11-20x as indicated in Fig. 2d.

The LOD for functional assays for both off- and on-resonance are listed in Table 1. The LODs obtained when the PC was on-resonance were between 1.9 ng/ml to 1.3 pg/ml, dependent upon the affinity of the analyte for its capture antibody. The LOD percentage change when the PC is on-resonance as compared to off-resonance is also listed in Table 1. Negative values indicate a reduced (improved) LOD. We found that the LODs were improved by 5-90% for 17 of the 18 different assays when the PC was on-resonance.

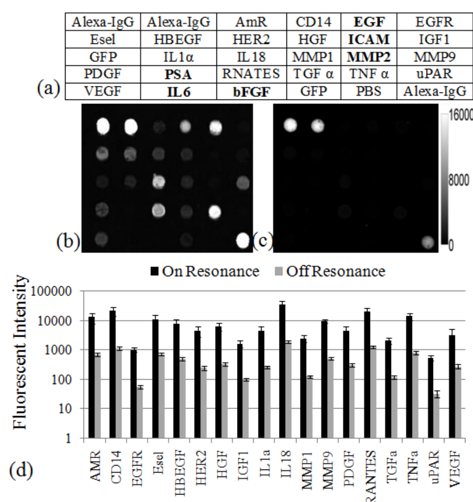

Fig. 2. (a) Layout of the capture antibodies within one protein microarray. Problematic assays highlighted by bold font. Fluorescence images of (b) PC on-resonance and (c) PC off-resonance. Both have the same fluorescent intensity scale shown in the left of (c). (d) Comparison of on-resonance and off-resonance measurements for each functional assay. Error bars represent the standard deviation of eight replicate spots.

Table 1. The LOD obtained in the experiment for off- and on-resonance. The LOD values are improved by 5-90% when the PC is on-resonance, with the exception of PDGF

Assay	LOD (pg/ml)		LOD % change
	Off-Resonance	On-Resonance	
AmR	320.0	206.8	-35.6
CD14	75.4	40.0	-48.4
EGFR	3858.6	846.4	-76.4
Esel	49.4	46.2	-6.5
HBEGF	10.4	6.9	-33
HER2	214.7	43.7	-79.6
HGF	108.3	11.8	-89.1
IGF1	1498	437.6	-70.7
IL1α	50.0	16.0	-68.0
IL18	6.5	2.7	-58.6
MMP1	764.3	107.6	-85.9
MMP9	130.1	19.2	-85.3
PDGF	8.3	9.9	19.3
RANTES	5.4	1.3	-75.4
TGFα	7.2	1.3	-81.5
TNFα	4.5	1.9	-57.3
uPar	7519.1	1954.5	-74.0

4. Conclusions

PCEF is demonstrated as an effective tool for reducing detection limits in the context of a cancer biomarker microarray. The resonant excitation effect increases the signal by 11-20x, resulting in reduction of the LOD by up to 90%. Dose-response characterization shows the capability to detect common cancer biomarkers in the 1.9 ng/ml to 1.3 pg/ml concentration range within a mixed sample.

5. References

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