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Multichannel Waveguides for the Simultaneous Detection of Disease Biomarkers.

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Abstract

The sensor team at the Los Alamos National Laboratory has developed a waveguide-based optical biosensor that has previously been used for the detection of biomarkers associated with diseases such as tuberculosis, breast cancer, anthrax and influenza in complex biological samples (e.g., serum and urine). However, no single biomarker can accurately predict disease. To address this issue, we developed a multiplex assay for the detection of components of the *Bacillus anthracis* lethal toxin on single mode planar optical waveguides with tunable quantum dots as the fluorescence reporter. This limited ability to multiplex is still insufficient for accurate detection of disease or for monitoring prognosis. In this manuscript, we demonstrate for the first time, the design, fabrication and successful evaluation of a multichannel planar optical waveguide for the simultaneous detection of at least three unknown samples in quadruplicate. We demonstrate the simultaneous, rapid (30 min), quantitative (with internal standard) and sensitive (limit of detection of 1 pM) detection of protective antigen and lethal factor of *Bacillus anthracis* in complex biological samples (serum) using specific monoclonal antibodies labeled with quantum dots as the fluorescence reporter.

Introduction: Several biomolecules specific to the pathogen of interest are secreted in an infected host. In malignant conditions such as cancer, many host molecules are differentially expressed during disease. Such 'biomarkers' can serve as very early and accurate indicators of disease. However, their application to disease diagnosis and detection has been limited for several reasons. First, biomarkers are often present in extremely low concentrations in the host and currently, specific ligands and sensitive technology for the detection of such markers are lacking. Second, non-specific interactions associated with detection in complex samples (e.g.; serum, urine, blood) is a significant problem. Third and perhaps most significant, no single biomarker can accurately detect and track disease. Hence, a suitable detection technology should be able to detect a suite of biomarkers in a single patient sample. Fourth, for biomarkers where changes in threshold concentrations are measured (e.g., host biomolecules upregulated during cancer), it is essential that the detection technology used be quantitative as well.

The sensor team at the Los Alamos National Laboratory has developed a waveguide-based optical biosensor and has previously been demonstrated to detect biomarkers associated with tuberculosis (*Mukundan H et al, Keystone, 2008*), anthrax (Martinez JS et al, 2005), breast cancer (*Mukundan H et al, BC and Mukundan H et al S&A, 2009*) and influenza (*kale RR et al, 2008*) in complex biological samples such as serum and urine. We have also demonstrated the feasibility of our approach for multiplex detection of two analytes on single mode planar optical waveguides using tunable quantum dots (QDs) as the fluorescence reporter (*Xie H et al, Submitted Angew Chemie, Mukundan H et al, Transactions of SPIE, 2009*). However, this limited multiplex capability is insufficient for

the effective diagnosis of diseases where quantitative, reproducible detection of a suite of biomarkers in replicate is desired.

In this manuscript, we present for the first time, the design, fabrication and development of a Multichannel waveguide for the simultaneous detection of three different samples in quadruplicate. The multi-element waveguide chip was adapted to our existing biosensor platform by the engineering of a suitable flow cell apparatus. The functional parameters of the waveguide were demonstrated by measuring the high-affinity binding of biotin to streptavidin across the 12 measurement elements. Ultimately, we demonstrate the feasibility of this approach to the quantitative detection of PA and LF in serum. These experiments have used photostable and tunable QDs as the fluorescence reporter. Also significant is the incorporation of a fluorescently labeled streptavidin as an internal control for the normalization of inter- and intra- assay variability.

Experimental:

Materials: Waveguides were fabricated at nGimat, Atlanta (see Methods). Purified antibodies (mouse monoclonal, IgG1, human adsorbed) that bind orthogonal epitopes of the anthrax PA (anti-PA antibody BAP106 for capture and BAP105 as reporter) were obtained from QED Bioscience Inc. Amino-functionalized QD (655 nm, QD655), Qdot®655 ITK™ carboxyl QDs and AF647-*N*-hydroxysuccinimide (NHS) ester labeling kits, streptavidin-QD conjugate (S-QD565) and 96 well plates were purchased from Invitrogen. Biotinylation, protein estimation and electrophoresis reagents were obtained from Pierce. Tri-n-octylphosphine oxide (TOPO) and tri-n-octylphosphorine (TOP) capped CdSe/ZnS core/shell QDs were a generous gift from Dr. J. Hollingsworth (Los Alamos National laboratory). Gel filtration columns and spin filters were from Harvard Apparatus. Buffers, chemicals, immunoblot materials and other components were purchased from Sigma Aldrich or Fisher Scientific. Materials required for waveguide functionalization are listed in detail elsewhere (*Anderson AS et al, 2008*).

Methods:

Design, Composition and Fabrication of the Multichannel Waveguide: The multichannel was designed based on specifications generated at Los Alamos National laboratory and generated at nGiMat, Atlanta. The waveguide consists of a 76. 2mm diameter, 2mm thick fused silica optically polished substrate (low refractive index, μ =1.46) and a Si_3N_4 high index film (μ =1.81), 110nm +/- 5nm thick. The very thin film requires the use of refractive grating elements for coupling light into the waveguide. The grating coupler elements are fabricated using a holographic interference technique to generate the periodic pattern (for a wavelength of 532nm and a +10 ° coupling angle the period is

420nm) prior to the deposition of the waveguide film. That pattern is etched into the substrate surface by a reactive ion etching process and subsequently over-coated with the Si_3N_4. To ensure the gratings are not affected by ambient refractive index changes, the grating coupler region must also be over-coated with a layer of dense SiO_2 (1100nm or thicker), thus isolating the evanescent field from the surrounding media. In addition to the Si_3N_4 waveguiding film, a thin (10 nm) SiO_2 film is deposited on the waveguide surface sensing area, which provides the functional surface required for the attachment of self-assembled monolayer or lipid bilayer sensing films. The multichannel waveguide consists of three channels with four sensing elements per channel (Figure 1 A and B). Each sensing element is 10mm long and 1.5mm wide and has a 1.5mm x 1.5mm detractive grating element, which enables each sensing element the ability to be excited independently of each other. The channels and sensing elements also have a chrome absorbing material surrounding them, allowing each element to be excited independently with out cross talk or inadvertent coupling between the sensing elements. After the waveguide coatings have been deposited onto the substrate, the substrate is cut to 76.2mm x 25.4 mm. Cutting the substrate to these dimensions permits it to be incorporated into a flow cell (specifically designed for these waveguides) and mounted into a holder for use on our waveguide-based optical biosensor (Figure 1B).

Functionalization of the Waveguide Surface and Flow Cell Assembly: The waveguide surface is functionalized using self assembled monolayers (SAMs) as is described in detail elsewhere (*Anderson AS et al, Langmuir, 2008*). The surface is biotinylated (0.1%) to facilitate the immobilization of a biotinylated capture antibody by biotin-avidin chemistry and allows for the use of a QD-labeled streptavidin (S-QD565) as the internal standard.

The functionalized waveguide is assembled in the flow cell that consists of a three channel (0.5mm thick) silicone rubber gasket and a glass cover with dimensions of 76.2mm x 25.4mm x 1mm with six holes precisely bored to align individual channels (Figure 1 B). The flow cell is mounted on a holder with septum material to form a seal with the glass cover and allow the addition of reagents *via* a syringe and be integrated into our experimental apparatus for measurement and detection. This configuration minimizes reagent volumes (100µl/channel), is self-sealing, and allows the addition of multiple reagents.

Fluorescence-based Sandwich Immunoassay on the Waveguide Surface:

a) Preparation of Antibodies: Antibodies that bind orthogonal epitopes of PA and LF were identified from either previous work in our laboratory *(Martinez JS et al, 2005)* or from the literature *(reference)*. The detailed protocols for the biotinylation of the capture antibody *(Martinez JS et al, 2005; Mukundan H Bioconj chem., 2009)* and QD labeling of the reporter antibody *(Mukundan H, Proc of the SPIE, 2009; Xie H et al, Agnew Chemie, submitted)* have been described elsewhere. Briefly, the capture antibodies were modified by biotinylation using sulfo-NHS biotin (20 molar excess) to allow their immobilization on the waveguide surface. The biotinylated antibodies (biotin-anti-PA or biotin-anti-LF) were characterized by HABA analysis for the degree of labeling and by immunoblot for functionality as previously described *(Mukundan H et al, Bioconjugate chemistry, 2009)* For QD labeling, carboxyl QD605 or QD655 stock (70 µl, 8 µM), diluted with 500 µl borate buffer (10 mM at pH 7.3) was mixed with 200 µg anti-PA or anti-LF antibody, respectively, in the presence of aqueous N-ethyl-N'-dimethylaminopropyl-carbodiimide and reacted at room temperature for 1.5 hours. The reaction was quenched by addition of borate buffer (500

μl, 50 mM at pH 9.0) and incubated at 4°C overnight, then washed five times with 1mL borate buffer (25 mM at pH 7.5) using 100 kDa molecular weight cut off (MWCO) spin filter and then passed through a 1000 kDa MWCO filter for clarification. Purified QD labeled antibodies (anti-PA-QD605 and anti-LF-QD655) were used as fluorescence reporters in the assay.

b) Measuring S-QD565 (Internal Standard) on the Waveguide-based Biosensor: For both standard and assay measurements, the flow cell was assembled as described before. No stabilization or blocking is required with the use of SAMs as the functional surface. A continuous wave 532nm diode-pumped solid-state (5 mW) frequency doubled Nd:YAG laser was used for excitation. In all experiments, the waveguide background (an intrinsic measure of impurities associated with the waveguide itself) and power coupled (input power = 440 μW) (Table 1), were measured and recorded in all three flow channels (referred to as A, B, C for the purposes of this manuscript). Injection volume was 200 μl and all incubations were for 10 minutes. Each injection is followed by a wash with PBS containing 0.5% BSA to remove unbound components.

S-QD565 (10-100 pM, depending on the experiment) was injected in all three-flow channels and the specific signal was measured (Table 1). Preliminary experiments were performed with streptavidin labeled with different QDs. We also determined that changing the direction of flow did not affect the distribution of sample (data now shown). For all experimental data included in this manuscript, the sample was injected in the orientation shown in figure 1B.

c) Multiplex Detection of PA and LF on the Multichannel Waveguide: For validation of sandwich immunoassays on the multichannel waveguide, the following experimental

matrix was used. Sandwich immunoassays for PA alone were performed in flow channel B with the S-QD565 internal standard, whereas that for LF alone were performed in flow channel C. Flow channel A alone was used for multiplex detection of both antigens in the presence of the standard.

In all experiments, unlabeled streptavidin (10 nM) was added to the flow cell after the S-QD565 measurement allowing for saturation of all biotin-binding sites on the functionalized waveguide. This was followed by addition of the biotinylated capture antibody (biotin-anti-PA and/or biotin-anti-LF, 80 nM each). Non-specific binding (NSB) associated with bovine serum and the fluorescently labeled reporter antibodies (anti-PA-QD605 and/or anti-LF-QD655, 100 nM each) was then determined in the absence of the antigen. For flow channel A, the reporter antibodies were added in sequence (first anti-PA-QD-605 and then anti-LF-QD-655), rather than together, to avoid any diffusional bias associated with the molecular mass of the conjugated QDs. Preliminary experiments were performed to show that the order of addition of the reporter antibodies did not change the observed signal (data now shown).

Subsequently, recombinant antigens (PA and/or LF) diluted in bovine serum were added to the flow cells in differing concentrations, depending on the experiment. Finally, the reporter antibodies were added and the specific signal associated with antigen binding was measured using the spectrometer interface.

Results and Discussion:

A multichannel waveguide capable to multiplex detection of three different samples in quadruplicate was successfully designed, fabricated and evaluated. The design and metrics of the waveguide are specified in Figure 1a and a photograph of the same

mounted on the specially designed flow cell is shown in Figure 1 b. In all experiments, the waveguide background (a measure of intrinsic impurities associated with the waveguide itself) and coupling efficiency (see below) was made. We also measured the binding of biotin (on the functional surface) to a QD-labeled streptavidin (S-QD565) in the 12 channels (Table 1). Input power was set at 440 μW for each sensing element. The coupling efficiency of a sensing element is measured as the ratio of the output power (measured with a power meter position perpendicular to the flow cell) to the input power (Table 1). This variation is reflected in the magnitude of the streptavidin-QD565 signal, which can thus be used as an internal standard to normalize for inter- and intra-assay variability. We performed several experiments where the injection port and outflow tube (Figure 1 b) were reversed. We found that this did not significantly affect the measured signal (data not shown). All data presented in this manuscript was generated in the orientation shown in Figure 1b. The design and fabrication of the multichannel waveguide allows for accurate detection in all 12 sensing elements without any fluorescence bleed-through. The injection time is very short (5 minutes) with respect to the diffusion time across the flow cell. Hence, diffusional impedance is not encountered in our flow cell design.

Multiplex immunoassays were performed on the multichannel waveguide for the simultaneous detection of PA and LF of the anthrax lethal toxin in serum. Figure 2 demonstrates the raw spectra observed for the simultaneous detection of 1 pM of PA and LF with the S-565QD control (10 pM) in a single grating of the multichannel waveguide. The waveguide-associated background is minimal (~100 RFU). The non-specific background associated with the LF antibody (2691 RFU) is much higher than that

observed with PA (405 RFU). Yet, the specific signal observed with antigen binding is higher (10619 RFU for LF and 703.2 RFU for PA) at 1 pM of each antigen. A signal/non-specific binding of 3.9 for LF and 1.75 for PA is observed. The limit of detection (LoD) of PA and LF on the multichannel waveguide in serum is thus 1 pM, which is significantly more sensitive than that observed in traditional plate-based immunoassays for these antigens and comparable to what we have previously seen on single channel planar optical waveguides for the same assay *(Xie H et al, Angew Chemie, 2009)*.

Figure 3 shows the measurement of the specific signal associated with PA or LF in each sensing element of a single multichannel waveguide. Waveguides are intrinsically different from each other with respect to their fabrication, ability to couple light and refractive index. Also, the functional properties of the waveguide vary depending on the efficiency of the SAM coating. Hence, the observed RFU are expected to be very different between waveguides (for e.g. figures 2 and 3). However, the LoD and the signal/noise are similar irrespective of the waveguide used, especially when the data is normalized to the internal standard (see below).

The signal is corrected for non-specific binding associated with control serum and the reporter antibody. As is apparent, there is significant disparity in the magnitude of the signal observed in the various elements. Figure 4 demonstrates the signal intensity normalized for the S-565QD internal standard. Using the internal standard successfully abrogates much of the variability of measurement in the different grating elements. However, some variability still exists in the normalized data, which may be a function of the SAM coating, flow or measurement conditions. We are currently working on

improving our flow cell design and coating protocols to overcome these variations. Figure 5 demonstrates the signal intensity observed with 1 pM PA and LF, normalized to S-565QD and averaged over the four grating elements in each flow channel. The ensemble measurement made in each flow channel (n=4) are not significantly different from that obtained in anther flow channel, validating the reproducibility of the data. This allows for reliable reproduction of the data within a single experiment and accommodates a greater confidence in a quantitatively meaningful result.

These results are but the first step towards the application of multichannel waveguides to multiplex biodetection of real-world samples. Currently, SAM deposition, gasket cutting and flow cell assembly are done manually requiring precision and skill. We anticipate automating these processes in the future to facilitate the easy adaptation of this technology to medical diagnostics.

Conclusions: No single biomarker can accurately detect disease. Therefore, the ability to detect a limited suite of biomarkers is quintessential to accurate biodetection. Reliability of detection also depends upon specificity (minimal non-specific interactions associated with complex samples and similar molecular entities), sensitivity (picomolar concentrations for most biomarkers) and reproducibility. For biomarkers where a change in threshold concentration is measured, it is also important for the assay to be quantitative. No single existing biodetection platform satisfies all these criteria. This has significantly limited the application of biomarkers to biomedical diagnostics.

In this manuscript, we have addressed some of these limitations in biomarker detection strategies using a Multichannel waveguide-based biosensor platform. The novel findings reported in this manuscript are 1) the design, fabrication and development of a multi-

channel waveguide and adapting it for use in our biosensor platform by engineering a flow cell (Figure 1), 2) adapting our SAM deposition protocols for such a waveguide, 3) validating the applicability of the waveguide for biodetection using a QD-labeled streptavidin (Figure 3, Table 1) and 4) validating the applicability of the waveguide to reproducible multiplex detection of anthrax lethal toxins in serum (Figures 4 and 5).

References:

Ho M; Pemerton J. *Anal Chem.* **1998**, *70*, 4915-4917.

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Legends:

Figure 1: Nile and Kevin, please rewrite this with an optical slant! Figure 1 A (top panel) is a photograph of the multichannel waveguide assembled in a flow cell. Laser light (532 nm excitation) is coupled into grating B2 and a 'streak' owing to propagation of the light by total internal reflection is seen. Figure 1 A (bottom panel) is a schematic representation of the multichannel waveguide (not to scale). Dimensions of the individual sensing elements are indicated. The cross bars indicate the grating etched into each sensing element. The gray bars in between the sensing elements represent a detractive grating element that allows each sensing channel to be measured independently without interference from a neighboring channel. Figure 1 B shows the multichannel waveguide assembled in a flow cell especially constructed for this purpose. The injection orientation and the flow channel designations are indicated.

Figure 2: The multiplex spectrum obtained in a single sensing element upon detection of S-565QD, PA and is shown. Black circles (•) represent the waveguide-background at that sensing element, which is a measure of intrinsic impurities associated with the waveguide itself. The triangles (Δ) indicate non-specific binding of control serum and the fluorescence reporter antibody to the functionalized waveguide surface in the absence of the antigen. Squares (□) indicate the specific signal associated with antigen (1 pM each)-reporter binding. A signal/noise of ~3.9 for LF and 1.75 for PA is observed under these conditions.

Figure 3: Maximal signal intensity observed at 676 nm in all 12 grating elements with the specific detection of 100 pM of PA/LF (or both) is indicated. The data is plotted as signal

intensity corrected for non-specific binding associated with the reporter antibody and control serum as a function of the waveguide channel. Both PA and LF were measured in channel A. Only PA was measured in channel B, whereas only LF was measured in channel C. Although a positive detection is achieved at this concentration in all12 sensing elements, there is a significant disparity in the signal observed.

Figure 4: Signal intensity from figure 3 is normalized to the internal standard signals (S-565QD) in this figure. A significant reduction in variability is achieved by normalization of the data to the standard.

Figure 5: Repetitive measurement of PA was made in channel A (sensing element 1 to 4) and channel B (sensing elements 1 to 4, top panel). For LF, similar measurements were made in channel A and C (Bottom panel). The average signal intensity from each channel is plotted. There is no significant difference in the observed data (student T-test, $p > 0.05$) validating the positive measurement (n=4).

Table 1: Summary measurement of the waveguide-associated background, coupling efficiency (uncoupled power is 440 μW in all channels) and the signal observed with 10 pM S-565QD (internal standard) is indicated for one multichannel waveguide.

Figure 1 A

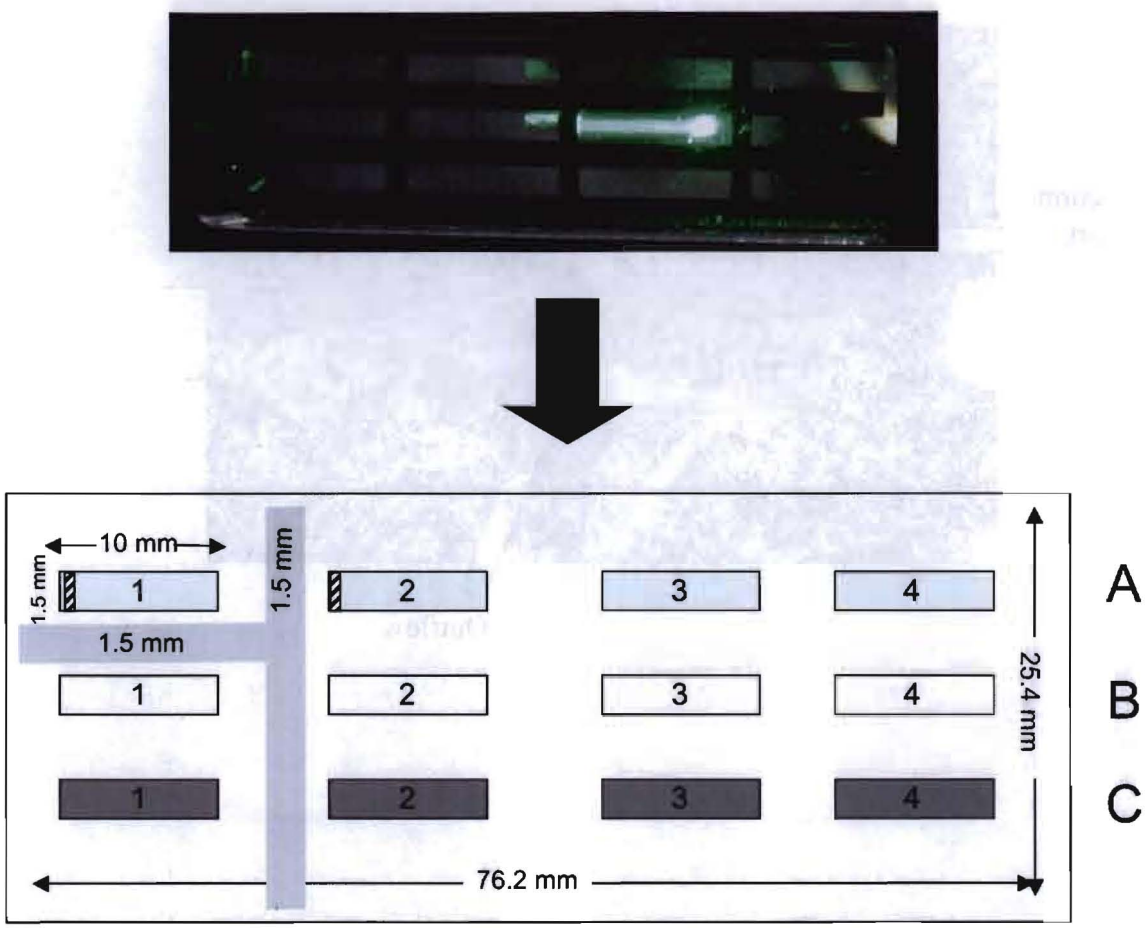

Figure 1 B

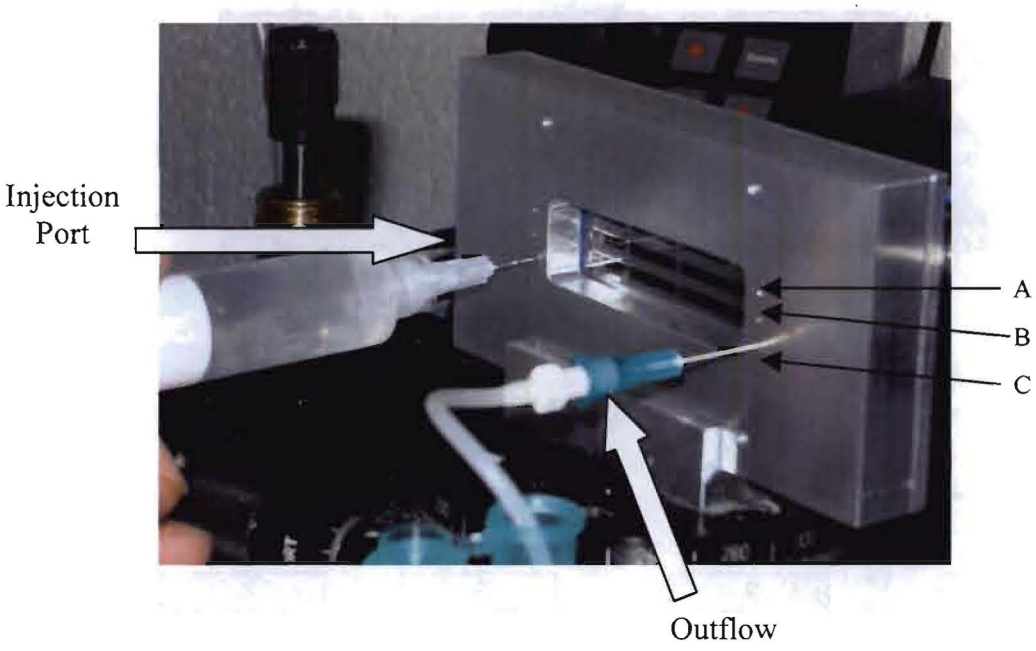

Figure 2

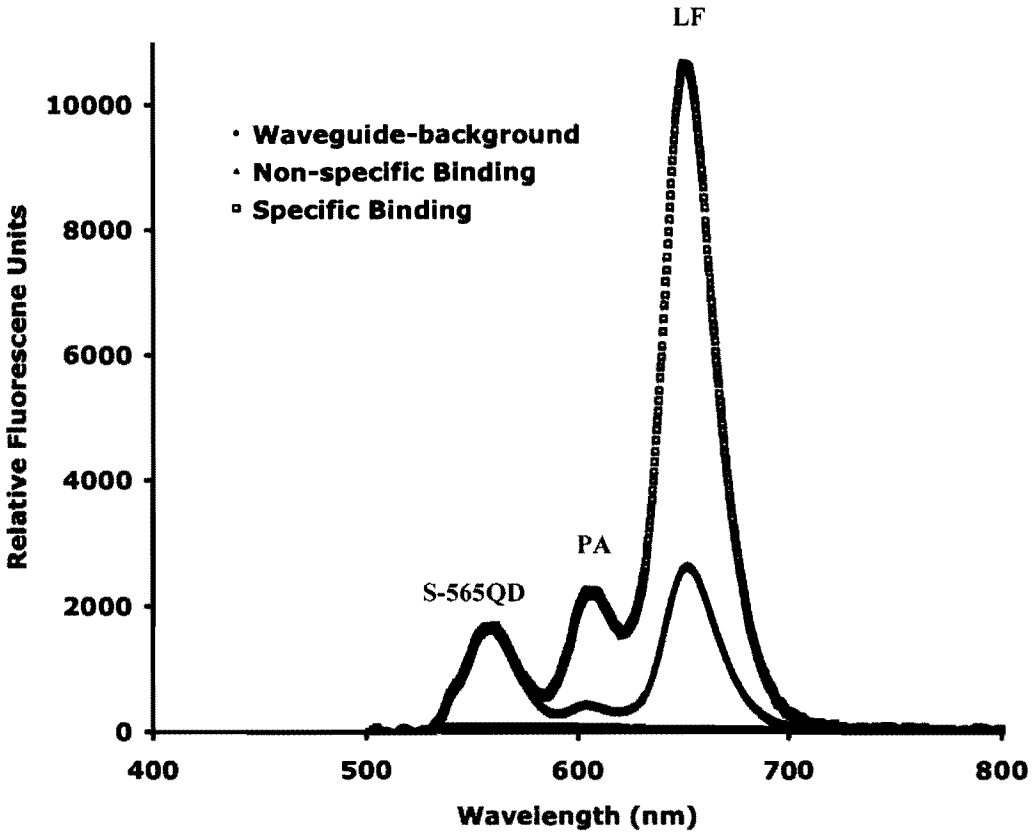
LF
10000
8000
6000
4000
2000
0
Relative Fluorescene Units
Waveguide-background
Non-specific Binding
Specific Binding
PA
S-565QD
400
500
600
700
800
Wavelength (nm)

Figure 3

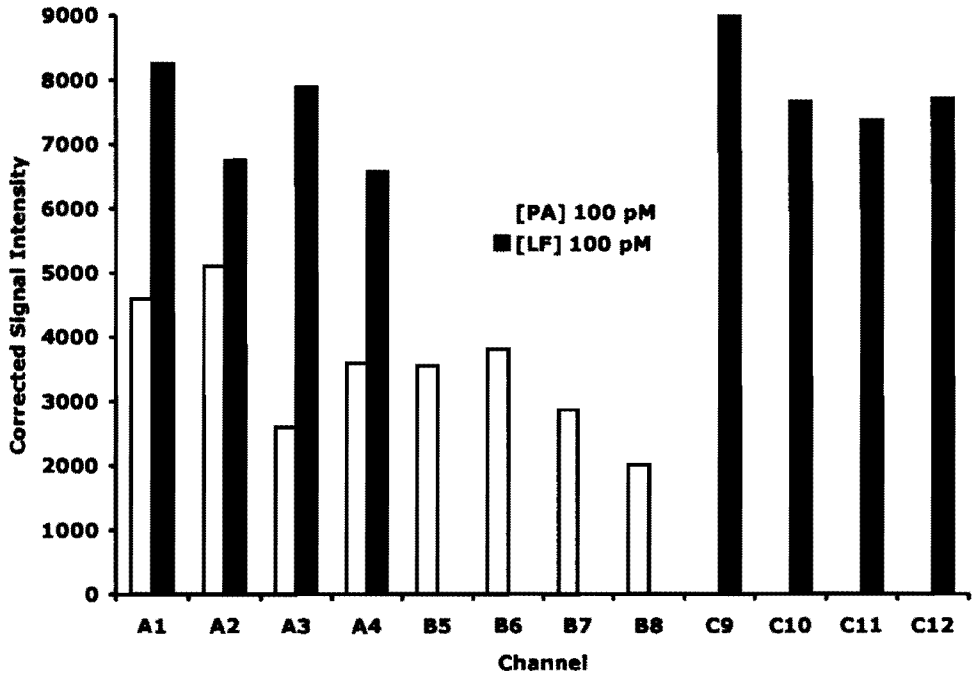
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
Corrected Signal Intensity
[PA] 100 pM
[LF] 100 pM
A1
A2
A3
A4
B5
B6
B7
B8
C9
C10
C11
C12
Channel

Figure 4

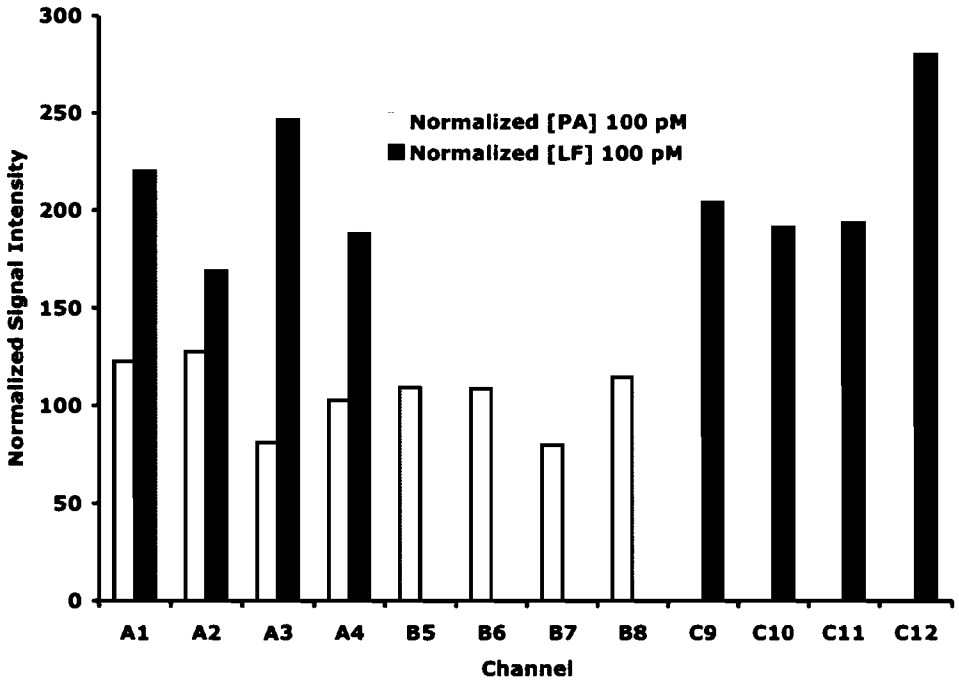
300
250
200
150
100
50
0
Normalized Signal Intensity
Normalized [PA] 100 pM
Normalized [LF] 100 pM
A1
A2
A3
A4
B5
B6
B7
B8
C9
C10
C11
C12
Channel

Figure 5

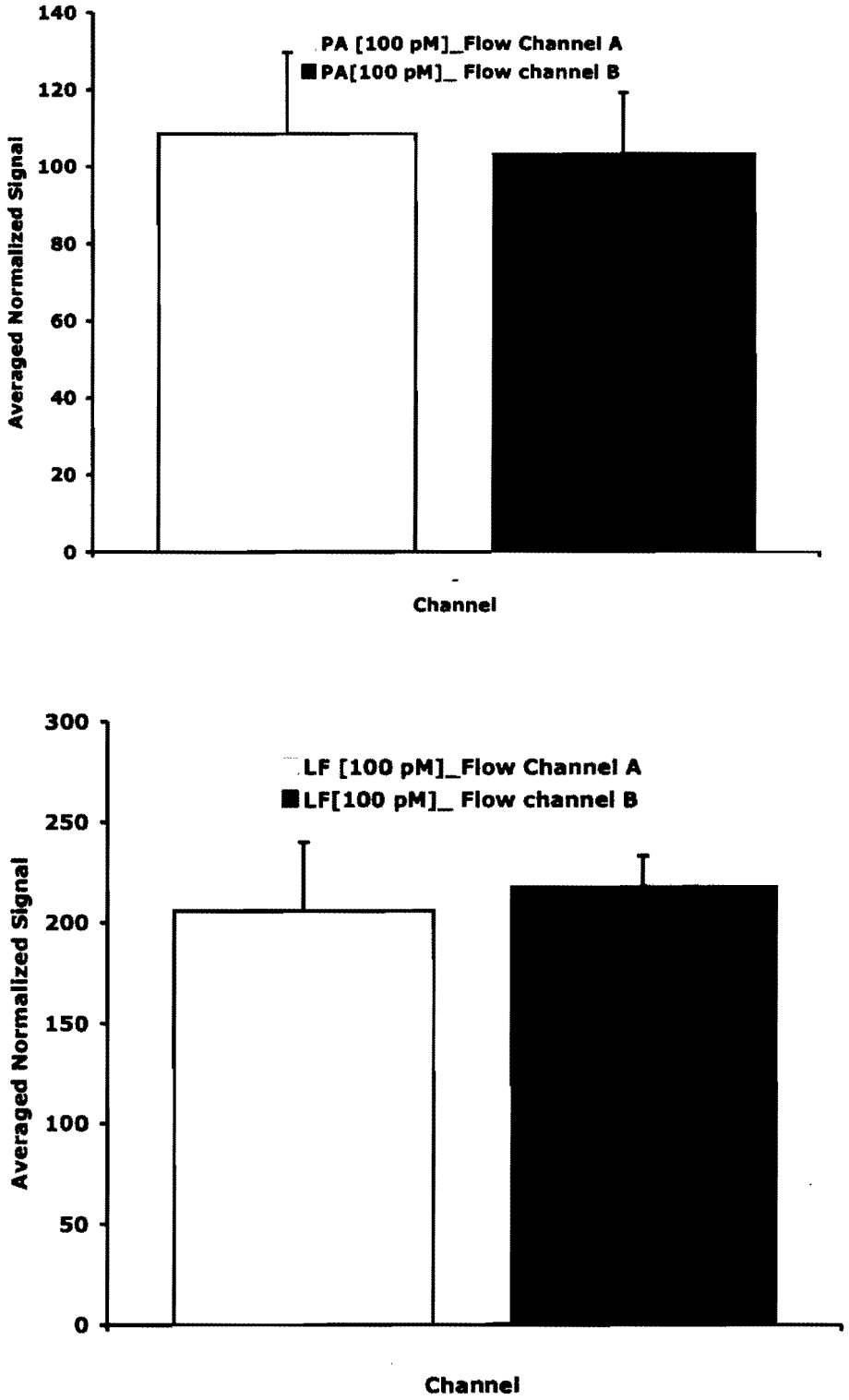
PA [100 pM]_Flow Channel A
PA[100 pM]_ Flow channel B
Averaged Normalized Signal
140
120
100
80
60
40
20
0
Channel
LF [100 pM]_Flow Channel A
LF[100 pM]_ Flow channel B
Averaged Normalized Signal
300
250
200
150
100
50
0
Channel

Table 1

Channel	Background	Power coupled (μW)	S565QD-10 pM
A1	125	179	3750
A2	225	140	5000
A3	175	160	3200
A4	190	195	2500
B5	180	170	3250
B6	210	155	3500
B7	250	155	3600
B8	150	210	1750
C9	190	194	2500
C10	200	150	4000
C11	300	175	3800
C12	240	210	2150