

body microarrays through capillary action. Fluid flow enhances the kinetic interaction between the analyte and the immobilized ligand thereby overcoming the diffusion limitation of the incubation assays and reducing assay time. Since the flow is over the arrays and not through a three dimensional matrix as in the case of LFAs, the arrays can be treated with blocking agents to minimize background noise. This provides a rapid, simple, yet multiplexed platform to measure protein biomarkers in serum samples.

A mixture containing sample, detector antibody and fluorescent reporter at predetermined concentrations was added to the protein array. Capillary forces directed this fluid into a chamber over the printed capture antibodies for binding. The fluid was then wicked from the other end of the channel using absorbent material. Flow rate through these capillary channels was controlled by choosing appropriate wicking material. This also ensures unidirectional flow of the sample. The protein biomarkers are quantified by an optical reader as the fluorescence of the spots is proportional to the analyte concentration. This technique enabled the rapid measurement of multiple protein biomarkers under flow conditions. The speed of the assay offers considerable advantages over more conventional antibody microarrays that require long incubation times.

To demonstrate the proof of principle of quantitation using the channels, biotin-BSA was immobilized in a series of 4 dilutions from 500  $\mu\text{g/ml}$  to 62.5  $\mu\text{g/ml}$ . Streptavidin QD at 10 nM was used as reporter. The arrays were imaged and the fluorescent intensities of the spots was quantified and plotted in the FIGS. 22(A and B, respectively). The spots show an increase in fluorescent intensity with increased concentration. A linear response was observed in this assay demonstrating the principle that quantitative standard curves can be obtained using the microarray channel flow device. To demonstrate the multiplexing capability, two different capture molecules were used in quadruplicate (shown as columns in the FIG. 22(C)); biotin-BSA at 500  $\mu\text{g/ml}$  and mouse IgG at 500  $\mu\text{g/ml}$ . BSA was used as the negative control. Both spots and were washed with a mixture of streptavidin QD 605 (10 nM) and Goat anti-mouse IgG-QD 605 (10 nM) for 2 min. Observed in the FIG. 22(C) are four sets of spots. In the first set on the left, no reporter was used and we see no signal. In the second set, both streptavidin QD 605 and Goat anti-mouse IgG-QD 605 were used and fluorescence is observed in both the biotin-BSA and Mouse IgG spots. In the third set, only streptavidin QD 605 and therefore only the biotin-BSA spots show fluorescence. In this final set, only Goat anti-mouse IgG-QD 605 is used resulting in fluorescence only in the Mouse IgG spots. This demonstrated the specificity of the microarray channels and its ability to measure two different analytes simultaneously.

The biotin-BSA and Mouse IgG spots were observed with "comet tails" or streaks in the direction of flow in the channel. Since this assay was designed purely for the demonstration of proof of principle, the concentrations of the reagents were not optimized. As a result, too much capture molecule was deposited onto the glass surface, resulting in excess unbound ligand, which bound to the fluorescent reporter molecules in solutions and were smeared on the glass surface generating a streaking effect. To optimize spot morphology and minimize streaking, capture molecule concentrations should be optimized.

#### Flow Channel Standard Curves with Quantum Dots

Standard curves were generated on flow channels by printing capture antibodies to the four protein biomarkers such that each antibody was present in quadruplicate within one channel. These arrays were incubated with 6 serial dilutions of

recombinant antigen diluted in human serum. Standard curves were obtained on the protein microarray format for each biomarker. FIG. 23 shows a composite image of six different arrays with each column representing a different array that was incubated with increasing antigen concentration from right to left. Slides shown in Panel A were incubated with Her-2 at concentrations ranging from 6.25 ng/ml (right) to 100 ng/ml (left). Slides shown in Panel B were incubated with MMP-2 with a concentration range of 62.5 ng/ml (right) to 1000 ng/ml (left). Slides shown in Panel C were incubated with Osteopontin at concentrations from 94 ng/ml (right) to 1500 ng/ml (left) and those shown in Panel D were incubated with CA 15-3 at concentrations ranging from 9.4 U/ml (right)-150 U/ml (left). Channels with no antigen added were treated as background. The results show increased fluorescence intensity with increased protein concentration for Osteopontin and CA 15-3. The fluorescence from these spots is quantified using the Scanarray software and plotted as a function of antigen concentration in FIG. 23 (E-F) for these experiments. The standard curves were observed to be linear for Osteopontin and CA 15-3 in the clinically-relevant ranges. However, no signal from either Her-2 or MMP-2 was observed.

To further investigate and confirm these results, a multiplex assay was performed in which all four protein biomarkers were analyzed in one single microarray. In this experiment, four identical channels were printed with capture antibodies to the four protein biomarkers. As shown in FIG. 24, one of these slides was incubated with only CA 15-3 antigen, the second slide was incubated with only Osteopontin antigen, the third slide with all antigens and the fourth with no antigen. All four biotinylated antibodies were used in each assay. While the results for the assays involving OPN and CA 15-3 yielded accurate and specific signals from the correct capture antibody spots, no signal was obtained from Her-2 and MMP-2 spots in the case where all antigens were added. This indicated that the assay for Her-2 and MMP-2 was not sensitive when a rapid, one-step technique was employed.

In order to troubleshoot the failure of Her-2 and MMP-2 detection on channels, sandwich assays on Her-2 and MMP-2 antigens were performed in three different formats. The first assay scheme was a "wash assay" which represented sequential incubation of assay reagents with washes in between, similar to a traditional microarray assay. The second scheme called "long assay" involved a one-step assay, where the antigen, biotinylated detector antibody and streptavidin QD 605 were premixed and incubated on the array for 60 min. The third assay scheme titled "short assay" was similar to the "long assay" except that the arrays were incubated for 30 min. In FIG. 25, the results from this test for Her-2 (A) and MMP-2 (B) are observed. Although the spots showed bright fluorescence for the wash assay, this signal was attenuated for the long assay in which no washes were included. This signal reached background levels for the short assay for both the antigens. The quantified signals are observed on the plot on the right hand side. This indicates that in order for the QD to be quantitative on the channel assays for Her-2 and MMP-2, they either need a long incubation time, or they need to be added individually to the arrays and cannot be pre-mixed with the sample. Therefore, Alexa 546 was adopted as the reporter for the channel assays since it had already been optimized in the above microarray assays.

#### Flow Channel Assays, Standard Curves Using Alexa 546

Standard curves were performed on all four antigens using streptavidin Alexa 546 as the reporter instead of QD 605. FIG. 26 (A-D) shows the quantified fluorescence from the array spots plotted as a function of antigen concentration. Channels