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Demonstration of Four Immunoassay Formats Using the Array Biosensor

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The ability of a fluorescence-based array biosensor to measure and quantify the binding of an antigen to an immobilized antibody has been demonstrated using the four different immunoassay formats: direct, competitive, displacement, and sandwich. A patterned array of antibodies specific for 2,4,6-trinitrotoluene (TNT) was immobilized onto the surface of a planar waveguide and used to measure signals from different antigen concentrations simultaneously. For direct, competitive, and displacement assays, which are one-step assays, measurements were obtained in real time. Dose−response curves were calculated for all four assay formats, demonstrating the array biosensor's ability to quantify the amount of antigen present in solution.

A variety of fluorescence-based array biosensors has been developed for performing multianalyte immunoassays. Most of these systems use either direct binding assays[1−5] or sandwich immunoassays,[6−11] but there have been a few reports of competitive immunoassays.[12−14] Displacement immunoassays, however, have only been reported using high surface area/volume systems.[15] The principle goal of this study was to demonstrate that the array biosensor could be used to perform all four types of immunoassays: direct, competitive, displacement, and sandwich.

We selected 2,4,6-trinitrotoluene (TNT) as the antigen for this demonstration for three reasons. First, TNT is univalent, i.e., it is a small molecule with only one epitope for antibody binding. However, 2,4,6-trinitrophenyl (TNP) derivates of TNT can be covalently bound to either a fluorophore for use in the direct, competitive, and displacement immunoassays or to a carrier protein to construct a multivalent antigen for performing the sandwich immunoassay. Thus, the same antibody and antigenic epitope can be used in all four immunoassay formats. Second, data are available for comparison in the published literature describing the sensitivity of other biosensor systems using TNT and anti-TNT antibody for both competitive and displacement immunoassays.[15−25] Third, the detection of TNT is a real problem for environmental monitoring and there is a current need for a rapid, selective, and reliable method for on-site quantification. The current standard protocol for TNT quantification in contaminated soil and groundwater samples is off-site laboratory analysis by reversed-phase HPLC.[26] On-site methods have been developed including both colorimetric tests and immunoassay kits such as ELISAs[17,27,28] and more recently a sol−gel-based biosensor;[29] with

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the exception of the displacement flow immunoassays,[17] these methods involve multistep procedures and take between 30 min and a few hours to process.

MATERIALS AND METHODS

Materials. Unless otherwise specified, chemicals were of reagent grade and used as received. The waveguides used in all the assays consisted of either custom-manufactured silver-clad slides[30] for real-time kinetic measurements or plain microscope slides (Daigger & Co Inc., Vernon Hills, IL) for the sandwich assay. The (3-mercaptopropyl)triethoxysilane was purchased from Fluka (AG, Buchs). Monoclonal antibody specific for TNT (A1.1.1) was purchased from Strategic Biosolutions (Ramona, CA; this antibody was previously known as IgG 50359 from Strategic Diagnostics). Biotinylated anti-TNT antibody was prepared by combining biotin-LC-NHS ester (Pierce, Rockford, IL) and the antibody in a 5:1 molar ratio and allowing them to react for 30 min. Biotinylated antibody was separated from unincorporated biotin using size exclusion chromatography (Bio-Gel P10, Bio-Rad, Hercules, CA). For the direct, competitive, and displacement assays, cyanine diaminopentane dihydrochloride-labeled trinitrophenyl (Cy5−DAP−TNP) was prepared, as described previously,[18] and TNT was purchased from Radian International (Austin, TX). For the sandwich assay format, fluorescently labeled anti-TNT antibody was prepared by reaction with Cy5 bisfunctional *N*-hydroxysuccinimide ester (Amersham Life Science Products, Arlington Heights, IL) following the manufacturer's instructions. Labeled antibody was separated from unincorporated dye using size exclusion chromatography. The dye-to-antibody ratio (mole/mole) was determined to be 3:1. The 2,4,6-trinitrobenzenesulfonic acid (TNBS) used in the production of the ovalbumin (OVA)−TNP complex was purchased from Pierce. Bovine albumin serum (BSA), OVA, and polyoxyethylenesorbitan monolaurate (Tween 20) were purchased from Sigma.

Patterning Antibodies Using Poly(dimethylsiloxane) (PDMS) Flow Cells. Both the plain and silver-clad glass slides were cleaned immediately prior to modification by immersion for 30 min in KOH (10% w/v) in isopropyl alcohol, followed by copious rinsing with deionized water and drying under a nitrogen stream. The cleaned slides were silanized under a nitrogen atmosphere by immersion for 1 h in an anhydrous toluene solution containing 2% (3-mercaptopropyl)triethoxysilane. The slides were then washed in anhydrous toluene and nitrogen-dried on a lint-free cloth, silver side up. The silanized slides were then immersed in a 1 mM *N*-(γ-maleimidobutyryloxy) succinimide ester (GMBS, Pierce, Rockford, IL), prepared by dissolving 12.5 mg of GMBS in 0.25 mL of DMSO and then diluting in 43 mL of absolute ethanol, for 30 min at room temperature. The waveguides were washed with water and incubated in 1.5 μM NeutrAvidin (Pierce) in 10 mM sodium phosphate/10 mM NaCl, pH 7.4 for 2 h at room temperature, washed in sodium phosphate buffer, and either used immediately for patterning with biotin-labeled anti-TNT antibody or stored at 4 °C in buffer.

Patterning of the antibody was carried out as previously described.[31] Briefly a six-channel PDMS (Nusil Silicone Technology, Carpintera, CA) flow cell was clamped onto the silvered surface of a waveguide that had previously been coated with NeutrAvidin. Biotin-conjugated anti-TNT antibody (20 μg mL^{-1} in 10 mM sodium phosphate/10 mM NaCl/0.05% Tween 20 solution) was introduced into the channels of the flow cell and incubated overnight at 4 °C on a rocker. Each channel of the PDMS flow cell had a width of 1500 μm, generating equal-sized columns of anti-TNT antibody on the waveguide surface.

Fluorescence Imaging and Regeneration. Each channel of the waveguide patterned with the anti-TNT antibody was rinsed with 1 mL of 10 mM sodium phosphate/10 mM NaCl/0.05% Tween 20 solution followed by 1 mL of 10 mM sodium phosphate/150 mM NaCl/0.05% Tween 20/1 mg mL^{-1} BSA (PBSTB) solution. The slide was removed from the patterning flow cell and immersed in 10 mM sodium phosphate/10 mM NaCl/10 mg mL^{-1} BSA for 20 min before being rinsed with PBSTB and attached to the on-line assay flow cell. The assay flow cell, used for real-time measurements, consisted of six channels (dimensions of each channel, 38 × 1.6 mm), machined in poly(methyl methacrylate) (Plexiglass G, Rohm and Haas, Philadelphia, PA) using a CNC mill, and a PDMS gasket.[32] The formation of fluorescent complexes on the waveguide surface was monitored using a CCD-based optical readout device, which has previously been described.[30] Briefly, evanescent wave excitation of the surface-bound fluorescent species was achieved using a 635 nm, 12-mW diode laser (Lasermax, Rochester, NY). A line generator was attached to the front of the laser for even distribution of illumination throughout the waveguide. The waveguide and attached flow cell were placed over the CCD camera and the laser light launched through a 1-cm focal length lens into the end of the waveguide at an appropriate angle.

Cy5−DAP−TNP (5 ng mL^{-1} in PBSTBE (PBSTB + 5% ethanol)) was passed over the surface of the waveguide once, at a flow rate of 2.67 mL min^{-1} using a Ismatec peristaltic pump (Cole-Parmer Instrument Co., Chicago, IL). The resulting fluorescence emission was monitored at right angles to the planar interface of the waveguide. A two-dimensional graded index of refraction (GRIN) lens array (Nippon Sheetglass, Summerset, NJ) was used to image the fluorescent pattern onto the Peltier-cooled CCD camera (Spectra Source, Teleris, Westlake Village, CA). Long-pass (Schott 0G-0665, Schott Glass, Duryea, PA) and band-pass filters (Corion S40-670-S, Franklin, MA) were mounted on the device scaffolding to eliminate scattered excitation light prior to CCD imaging. The CCD image of the waveguide was recorded, with an exposure time of 0.75 s, every 30 s over the first 3 min then at 4 and 5 min. After completion of the assay, the waveguide was regenerated with 10 mM sodium phosphate/10 mM NaCl/50% ethanol for 5 min, with the CCD image recorded at 2.5 and 5 min, followed by a 2-min wash with PBSTBE, with 1- and 2-min CCD images taken, before the cycle was repeated.

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Real-Time Assays. For the direct assay, a silver-clad slide was prepared and assembled in the flow cell as described in the previous section. Each channel was exposed to a different concentration of Cy5−DAP−TNP in PBSTBE, ranging from 0 to 10 ng mL^{-1}, with a flow rate of 2.67 mL min^{-1}. The CCD image was taken every 20 s over the 5-min time period of the assay, using an exposure time of 0.5 s. The slide was then exposed to the regeneration buffer for 5 min followed by a 2-min wash with PBSTBE. A final CCD image of the slide was recorded before the next assay.

For the competitive assay, each channel of the newly regenerated slide was exposed to different solutions, each containing 7.5 ng mL^{-1} Cy5−DAP−TNP in PBSTBE and various concentrations of TNT ranging from 0 to 200 ng mL^{-1}, at a flow rate of 2.67 mL min^{-1}. The CCD image of the slide was recorded every 20 s throughout the duration of the 5-min assay, with an exposure time of 0.5 s. In preparation for the displacement assay, the slide was then exposed to the regeneration buffer for 5 min followed by a 2-min wash with PBSTBE, before the final CCD image was recorded.

For the displacement assay, each channel was initially exposed to 7.5 ng mL^{-1} of Cy5−DAP−TNP in PBSTBE for 10 min and the CCD image monitored every 2.5 min. After the final CCD image was recorded, each channel was exposed to a different concentration of TNT, ranging from 0 to 200 ng mL^{-1}, at a flow rate of 2.67 mL min^{-1}. The CCD image was measured every 20 s over the 5-min assay period, with an exposure time of 0.5 s.

Sandwich Assays. The OVA−TNP complex used in the sandwich assays was prepared in the following manner. First, 0.625 g of OVA was dissolved in 25 mL of borate buffer (0.5 M Na borate/0.04 M NaCl, pH 8.5) and 0.404 mL of TNBS solution added (OVA/TNP ratio, 1:5). This mixture was then left to stir overnight at 4 °C wrapped in aluminum foil. TNP-labeled OVA was separated from unincorporated TNBS using size exclusion chromatography (Bio-Gel P10, Bio-Rad), and all fractions with absorbance at both 280 and 340 nm were combined. The OVA−TNP complex was stored until required at 4 °C.

Since the polyvalent TNT antigen was not fluorescent, the binding could not be measured in real time; thus, only the final CCD image of the slide after completion of the assay was recorded. An unclad microscope slide was patterned with the anti-TNT antibody and rinsed with 1 mL of 10 mM sodium phosphate/10 mM NaCl/0.05% Tween 20 solution followed by 1 mL of PBSTB solution. The slide was removed from the patterning flow cell and immersed in a 10 mM sodium phosphate/10 mM NaCl/10 mg mL^{-1} BSA for 20 min before being rinsed with PBSTB and assembled in the six-channel PDMS flow cell. Each channel was initially washed with 1 mL of PBSTB to check the flow cell for leaks and then exposed to various concentrations of OVA−TNP ranging from 0 to 6.5 μg mL^{-1} (equivalent to 0−150 ng mL^{-1} TNP) under static conditions for 5 min before each channel was washed with 1 mL of PBSTB. The slide was then exposed to 20 μg mL^{-1} Cy5−anti-TNT antibody for 10 min before being washed with 1 mL of PBSTB followed by 1 mL of water. The flow cell was removed and the slide dried with nitrogen and imaged using an exposure time of 2 s.

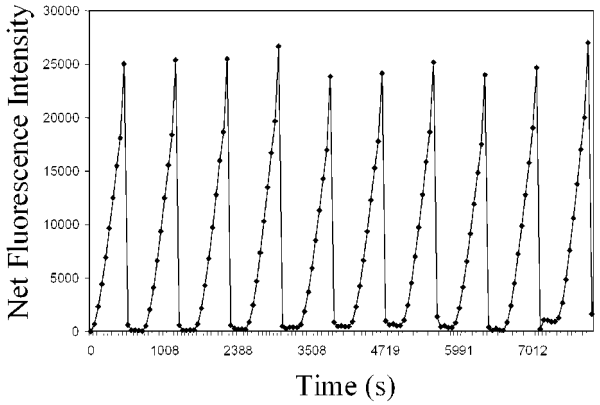

Figure 1. Antibody regeneration of the planar waveguide surface. A plot of the net fluorescence intensity for a central sensing spot on the waveguide over time. The waveguide was exposed to repeated cycles of 5 ng mL^{-1} Cy5−DAP−TNP (5 min)/regeneration buffer (5 min)/PBSTBE (2 min) a total of 10 times.

Data Analysis. Data analysis was preformed as described previously.[5] Briefly, to analyze the images, custom software was written in Lab Windows/CVI (National Instruments). A virtual mask was created using the final CCD image of the slide exposed to 7.5 ng mL^{-1} Cy5−DAP−TNP, prior to the displacement assay, as every sensing spot was illuminated. This mask could be used to analyze the direct, competitive, and displacement assay data sets because the waveguide was not moved between these different assays. A separate mask was created for the sandwich assay data set. After the software analyzed all the files in the data set, an Excel file was automatically created containing values of the time, mean intensity in the data area, mean intensity in the background region, and net intensity (data region minus background). The background signal was a measurement of the mean fluorescence from regions 10 pixels to either side of the data area. The data were further analyzed with SigmaPlot regression library to evaluate dose−response curves.

RESULTS AND DISCUSSION

Antibody Regeneration. Fiber-optic studies have shown that the removal of TNT and Cy5-EDA-TNP antigens bound to an anti-TNT antibody (11B3) was possible, without denaturation of the antibody, due to the low affinity of the antibody for the antigens.[22] Regeneration was achieved by introducing buffers containing high ethanol concentrations, typically 50%. To determine whether regeneration of the Strategic Biosolutions anti-TNT antibody immobilized onto a planar surface was also possible, a waveguide was exposed to repeated cycles of antigen/regeneration buffer/PBSTBE wash under direct binding conditions using Cy5−DAP−TNP. Figure 1 displays 10 cycles of antigen exposure followed by regeneration. It can be seen that there are only slight variations, CV 4.2%, in the maximum fluorescence intensity reached after each 5-min exposure to 5 ng mL^{-1} Cy5−DAP−TNP, with no apparent decrease in antibody activity. The ability to regenerate the antibodies is important not only from a commercial viewpoint but also because it allows the direct comparison of the different assay formats as the same antibody sensing regions are use for each assay.

Direct Immunoassay. The direct immunoassay is the simplest method to perform and involves the direct binding of the

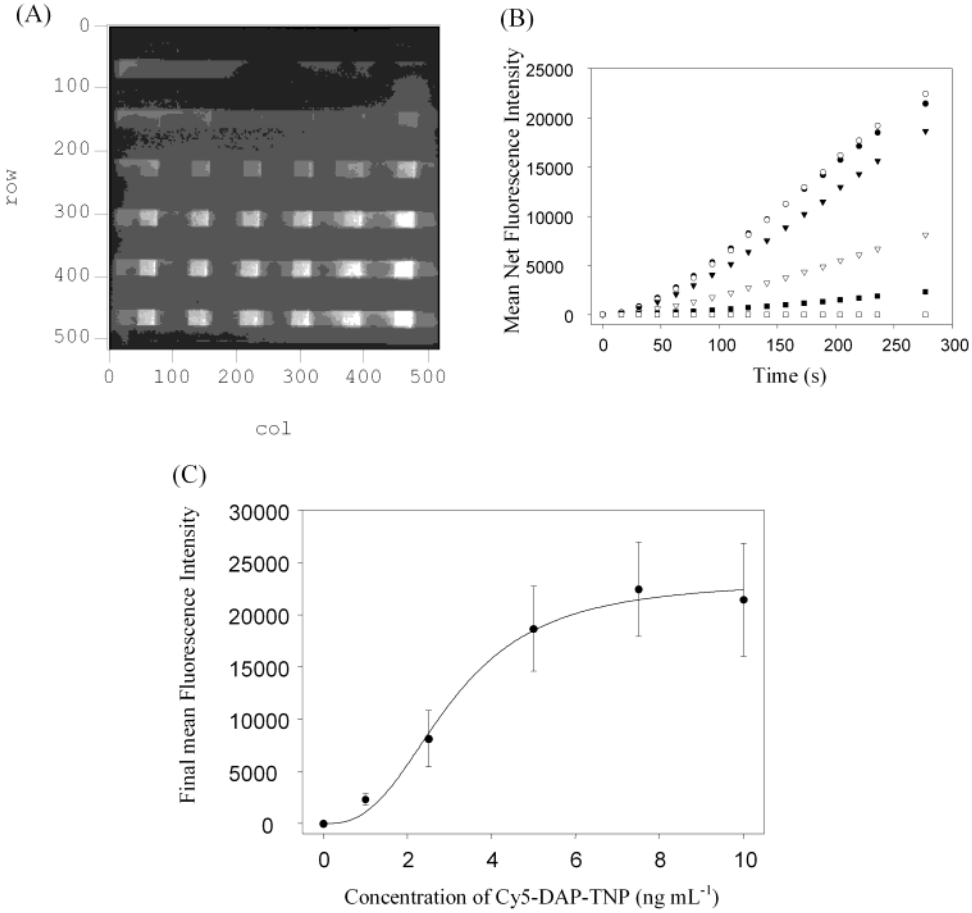

Figure 2. Direct assay format. The waveguide was exposed to various Cy5−DAP−TNP solutions ranging in concentration from 0 to 10 ng mL^{-1}. The final CCD image after the direct assay is shown in (A). The *x* and *y* axes in (A) represent the 25 × 25 μm pixel number in the corresponding column (col) or row, respectively. The resulting real-time binding of Cy5−DAP−TNP to the surface-immobilized antibody is shown in (B) as a plot of mean net fluorescence intensity (the mean of the six spots in the row; CVs for these points average at 12.7%) versus time for 0 (open square), 1 (dark square), 2.5 (open triangle), 5.0 (dark triangle), 7.5 (open circle), and 10 (dark circle) ng mL^{-1} Cy5−DAP−TNP. Plot C shows the resulting dose−response curve as a plot of maximum fluorescence intensity reached after completion of the 5-min assay versus Cy5−DAP−TNP concentration.

antigen to the immobilized antibody. However, it requires that the antigen contain some form of fluorescence, either intrinsic or via a fluorescent label. Hence, in order to carry out the direct assay, the TNT derivative TNP was labeled with Cy5 to form the fluorescent complex Cy5−DAP−TNP.

A silvered slide was assembled in the six-channel flow cell, and various concentrations of Cy5−DAP−TNP were flowed continuously over the sensor surface, to maintain reaction-rate-limited kinetics throughout the 5-min assay. The final CCD image, after completion of the immunoassay, is shown in Figure 2A. Each row of six sensing spots, representing one channel in the flow cell, was exposed to one specific concentration of Cy5−DAP−TNP, ranging from 0 ng mL^{-1} in row 1 (top of the image) to 10 ng mL^{-1} in row 6 (bottom of the image). The image shows the increasing fluorescence intensity from rows 0−6 as the concentration of Cy5−DAP−TNP increases. The increase in fluorescence intensity is more clearly demonstrated in Figure 2B which shows the mean net fluorescence increase in each row as a function of time. Each data point on a particular curve represents the mean of all the six spots contained in that row, with an average CV of ∼12%. The rise in fluorescence intensity appears to follow an asymmetric sigmodal function. The dose−response curve is plotted in Figure 2C as the final mean fluorescence intensity reached, after the 5-min assay, versus Cy5−DAP−TNP concentration. The data were fitted to a three-parameter linear log function (asymmetric sigmoidal) using SigmaPlot and found to have the equation $Y = 23290/[1 + (x/3.04)^{-2.71}]$ with $R^2 = 0.993$. The lowest measured concentration, discernible above the mean ± 3SD of the blank, was 1 ng mL^{-1} Cy5−DAP−TNP. The dynamic range of the response was between 1 and 8 ng/mL; above 8 ng mL^{-1}, the sigmoidal dose−response curve begins to plateau.

The direct assay, although the simplest to perform, requires that the antigen contain some intrinsic fluorescence. In the absence of such intrinsic fluorescence, other assay formats such as competitive, displacement, and sandwich are preferred.

Competitive Immunoassay. In the competitive assay format, a fluorescently labeled antigen competes with a nonlabeled antigen for binding sites on the immobilized antibody. This assay format is especially useful in the detection of small molecules that are not large enough to possess two distinct epitopes and has been used successfully for the detection of TNT using the fiber-optic biosensor.[21−24] After completion of the direct assay, the antibody sensing surface was regenerated using the buffer containing 50% ethanol. Solutions containing a fixed concentration of Cy5−DAP−

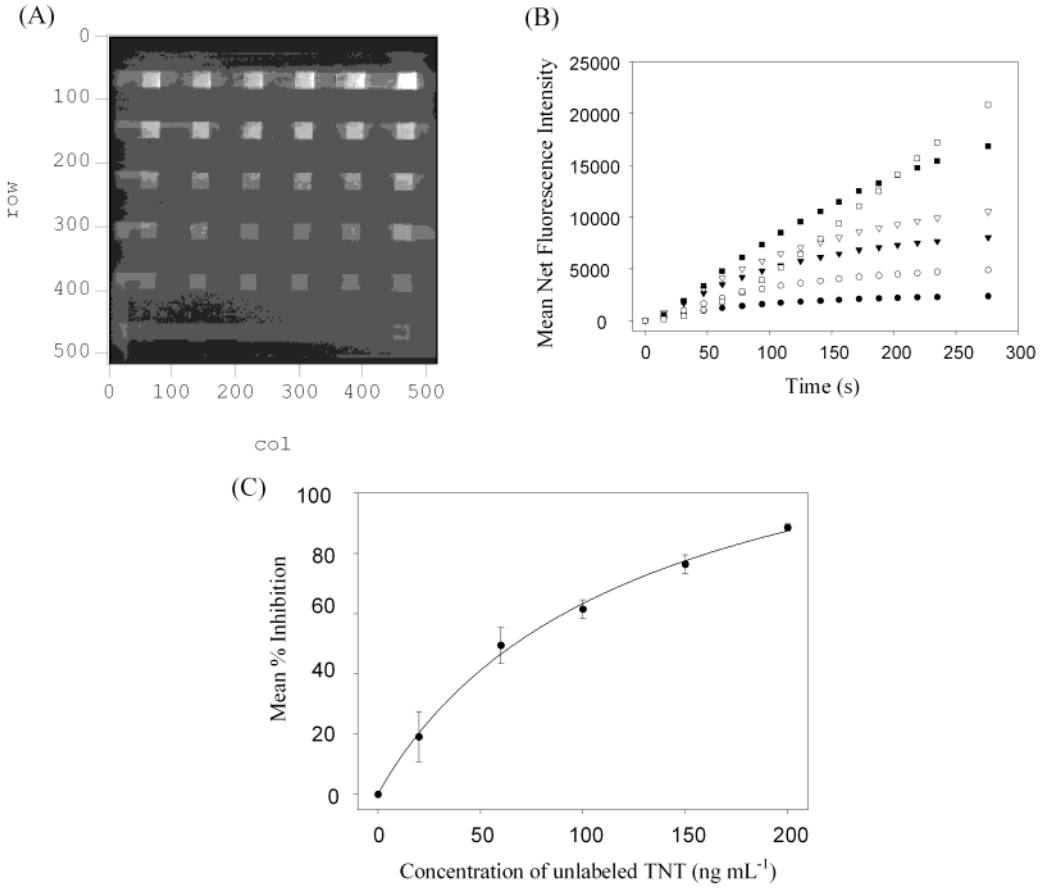

Figure 3. Competitive assay format. The waveguide was exposed to solutions containing a fixed Cy5−DAP−TNP concentration of 7.5 ng mL^{-1} and various concentrations of unlabeled TNT ranging in concentration from 0 to 200 ng mL^{-1}. The final CCD image after the competitive assay is shown in (A). The *x* and *y* axes in (A) represent the 25 × 25 μm pixel number in the corresponding column (col) or row, respectively. The resulting real-time binding of Cy5−DAP−TNP to the surface immobilized antibody, while in competition with unlabeled TNT, is shown in (B) a plot of mean net fluorescence intensity (the mean of the six spots in the row; CVs for these points average at 10.0%) versus time for 0 (open square), 20 (dark square), 60 (open triangle), 100 (dark triangle), 150 (open circle), and 200 (dark circle) ng mL^{-1} unlabeled TNT. Plot C shows the resulting dose−response curve as a plot of mean percent inhibition after completion of the 5-min assay versus the unlabeled TNT concentration.

TNP (7.5 ng mL^{-1}) and various concentrations of unlabeled TNT were flowed continuously over the sensor surface for 5 min. The final CCD image, after completion of the competitive immunoassay, is shown in Figure 3A. Each row of six sensing spots, representing one channel in the flow cell, was exposed to Cy5−DAP−TNP plus one specific concentration of unlabeled TNT, ranging from 0 (row 1) to 200 ng mL^{-1} (row 6). The image shows the expected decrease in fluorescence intensity, due to Cy5−DAP−TNP binding, as the concentration of the unlabeled TNT increases. This clearly demonstrates competition between the labeled and unlabeled antigens for binding sites on the immobilized antibody.

The mean net fluorescence intensity plotted as a function of time is shown in Figure 3B. Each data point on each curve represents the mean of all six spots contained in a particular row, with an average CV of ~10%. The dose−response curve is plotted in Figure 3C as the mean percent inhibition, determined using eq 1, versus the concentration of unlabeled TNT present in solution. The data were fitted to an asymmetric sigmoidal curve resulting in the equation of fit: $Y = 145.8/[1 + (x/131.3)^{-0.96}]$ with $R^2 = 0.997$. The lowest measured concentration, discernible above the mean ± 3SD of the blank, was 20 ng mL^{-1} unlabeled TNT; this is higher than that measured for either the fiber-optic biosensor (5 ng mL^{-1}),[24] the competitive ELISA (0.06 ng mL^{-1}),[25] or the chemiluminescent biochip sensor (0.13 ng mL^{-1})[33] all using the same A1.1.1 anti-TNT antibody for antigen capture. The dynamic range of the response was between 20 and 200 ng mL^{-1}. Although not measured above 200 ng mL^{-1}, the sigmodal dose−response curve does appear to be approaching the 100% plateau.

$$\%\ \text{inhibition} = \left(1 - \frac{(\text{final fluorescence intensity of sample})}{(\text{final fluorescence intensity with no unlabeled TNT})}\right) \times 100 \quad (1)$$

Displacement Immunoassays. The displacement assay format requires the initial saturation of all the antibody binding sites with the fluorescently labeled antigen. Upon introduction of the unlabeled antigen, displacement of the labeled antigen occurs and is measured in this sensor as a decrease in the fluorescence intensity. After completion of the competitive assay, the antibody sensing surface was regenerated and all the sensing spots were exposed to 7.5 ng mL^{-1} Cy5−DAP−TNP for 10 min in order to saturate the antibody binding sites with fluorescently labeled

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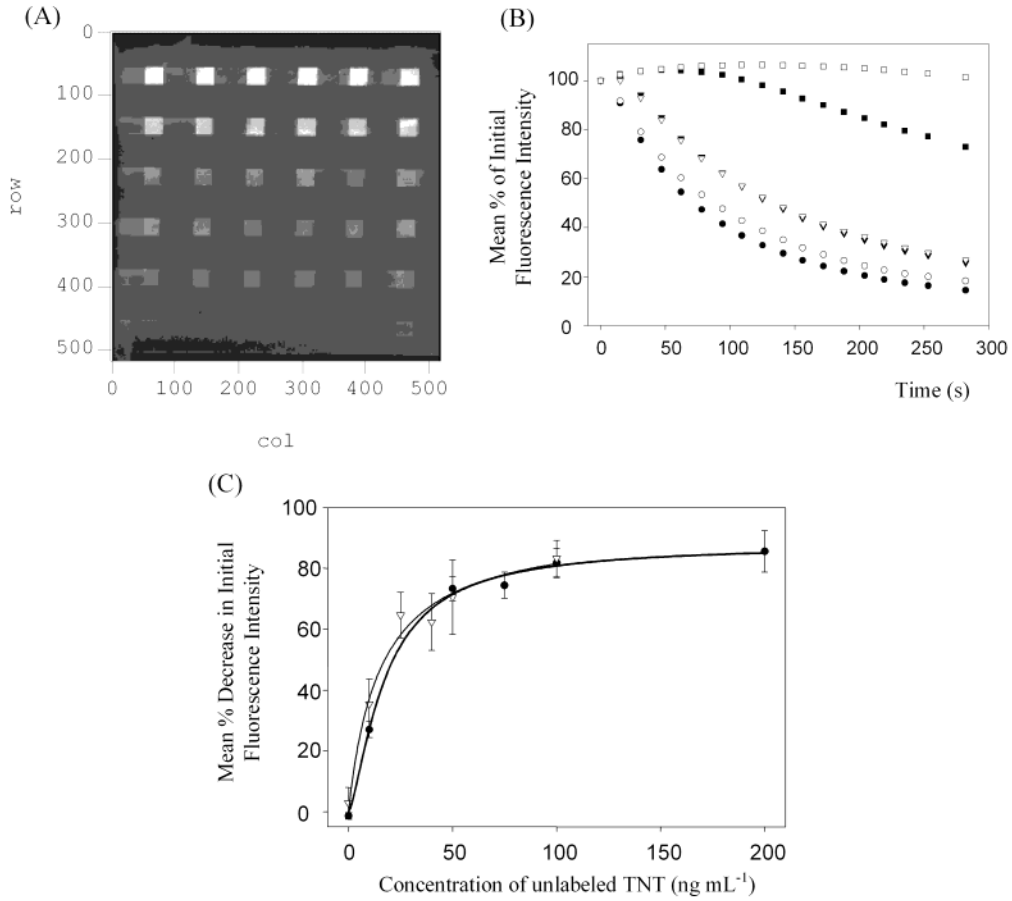

Figure 4. Displacement assay format. The waveguide was first exposed to 7.5 ng mL^{-1} Cy5−DAP−TNP for 10 min. The slide was then exposed to various concentrations of unlabeled TNT. The final CCD image after the displacement assay is shown in (A). The *x* and *y* axes in (A) represent the 25 × 25 μm pixel number in the corresponding column (col) or row, respectively. The resulting real-time displacement of Cy5−DAP−TNP from the surface-immobilized antibody by unlabeled TNT is shown in (B) as a plot of mean net fluorescence intensity (the mean of the six spots in the row; CVs for these points average at 13.4%) versus time for 0 (open square), 10 (dark square), 50 (open triangle), 75 (dark triangle), 100 (open circle), and 200 (dark circle) ng mL^{-1} unlabeled TNT. Plot C shows the resulting dose−response curve as a plot of the mean percent decrease in the fluorescence intensity after completion of the 5-min assay versus the unlabeled TNT concentration (dark circles). The second curve (open triangles) shows data, taken over a narrow concentration range, from a second displacement assay carried out on a waveguide prepared in a different batch from the previous one.

antigen. Various concentrations of unlabeled TNT were then flowed continuously, once, over the sensing surface during the 5-min assay (total of 13.5 mL of antigen solution). The final CCD image, after completion of the immunoassay is shown in Figure 4A. Each row of six sensing spots, representing one channel of the flow cell, was exposed to one specific concentration of unlabeled TNT ranging from 0 (row 1) to 200 ng mL^{-1} (row 6). The image shows the resulting decrease in fluorescence intensity between rows 1 and 6 as the concentration of unlabeled TNT increases. This clearly demonstrates the displacement of fluorescently labeled Cy5−DAP−TNP from antibody binding sites.

The decrease in the fluorescence intensity, calculated using eq 2, is plotted versus time in Figure 4B and shows the resulting

$$\%\ \text{of the initial fluorescence intensity} = \left(\frac{\text{fluorescence intensity at } t = x}{\text{fluorescence intensity at } t = 0}\right) \times 100 \quad (2)$$

displacement curves. Each data point on a particular curve represents the mean of all six spots in that row, with an average CV of ∼13%. Although 10 ng mL^{-1} unlabeled TNT clearly causes displacement of Cy5−DAP−TNP after completion of the 5-min assay, there is an initial time delay before displacement occurs, which is found to a lesser extent as the concentration of unlabeled TNT increases.

The dose−response curve of the assay performed as in Figure 4A and B is plotted in Figure 4C (dark circles) as the percentage decrease in the initial fluorescence intensity as a function of the unlabeled TNT concentration. Due to the initial rapid increase in the dose−response curve, a second displacement assay was carried out on a slide prepared in a different batch from the previous slide, over a slightly narrower concentration range of 0−100 ng mL^{-1} unlabeled TNT. The dose−response curve for this slide is plotted in Figure 4C as open triangles. Both the curves were fitted with an asymmetric sigmoidal using SigmaPlot and gave the following equations, $Y = 87.8/[1 + (x/17.6)^{-1.41}]$ with $R^2 = 0.997$ and $Y = 94.4/[1 + (x/15.5)^{-0.99}]$ with $R^2 = 0.979$, respectively. It can be seen from Figure 4C that although the slides were prepared in different batches, the data are highly comparable. The lowest measured concentration, discernible above the mean

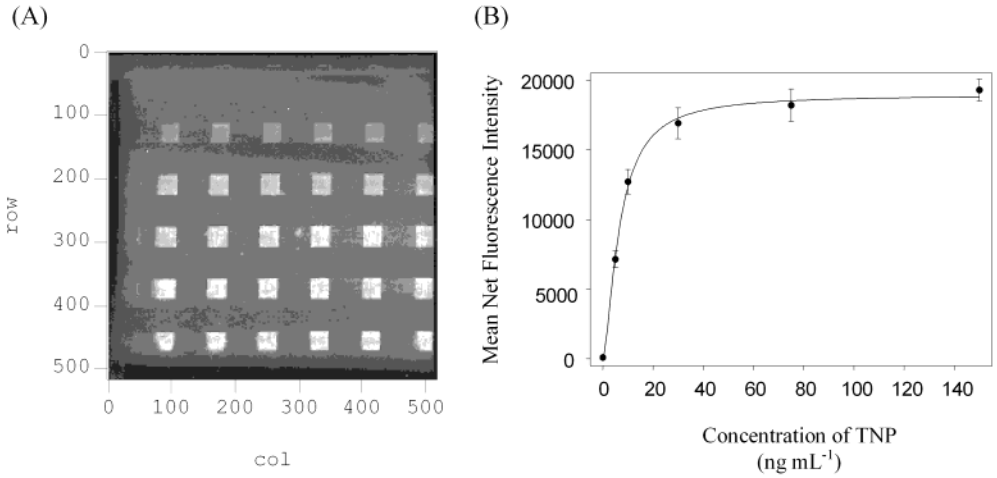

Figure 5. Sandwich assay format. The waveguide was first exposed to various concentrations of OVA−TNP ranging in concentration from 0 to 6.5 μg mL^{-1} for 5 min. The slide was then exposed to 20 μg mL^{-1} Cy5−anti-TNT antibody for 10 min before imaging. The final CCD image after the sandwich assay is shown in (A). The *x* and *y* axes in (A) represent the 25 × 25 μm pixel number in the corresponding column (col) or row, respectively. Plot C shows the resulting dose−response curve as a plot of mean maximum fluorescence intensity reached after completion of the 5-min assay versus the TNP concentration.

± 3SD of the blank, was 10 ng mL^{-1} unlabeled TNT. This is higher than the 0.1 ng mL^{-1} limit of detection reported by Narang et al. using a capillary-based displacement immunoassay with the same A1.1.1 anti-TNT antibody.[15] The dynamic range of the response was between 10 and 50 ng mL^{-1}; above 50 ng mL^{-1}, the sigmoidal dose−response curve reaches a plateau.

Sandwich Immunoassay. Sandwich assays require relatively large antigens that contain at least two epitopes for antibody binding. The antigen is bound to the immobilized capture antibody at one epitope and is detected using a fluorescently labeled tracer antibody bound to a second epitope. TNT as a small molecule is unsuitable for detection using the sandwich assay format. This feature was confirmed in a control assay in which TNT was bound to the capture antibody and Cy5−anti-TNT antibody then passed over the surface; the final CCD image showed no detectable fluorescence (data not shown). Therefore, an analogue of TNT, TNBS was complexed with the protein OVA, such that when the OVA−TNP complex bound to the capture antibody, the tracer antibody could interact with a separate TNP on another part of the same OVA−TNP complex.

Sandwich assays were initially carried out on the regenerated slide from the displacement assay. However, no detectable fluorescence could be measured at the end of the real-time assay. Yet when the assay was carried out under static conditions, fluorescence was easily detectable. It was later determined that the relatively hydrophobic OVA−TNP complex was sticking to the silicone tubing that transported the sample to the flow cell in the automated sandwich immunoassay (data not shown). Thus, the OVA−TNP complex was not reaching the sensing surface.

Therefore, the OVA−TNP sandwich assay was performed under static conditions with samples and tracer antibody added directly to the flow channels over the sensing surface. The final image after completion of the assay is shown in Figure 5A. Each row of six sensing spots was exposed to one specific concentration of the OVA−TNP complex, ranging from 0 (row 1) to 6.5 μg mL^{-1} (row 6) (which is equivalent to 0−150 ng mL^{-1} TNP, assuming 5 TNP per OVA−TNP complex), followed by 20 μg mL^{-1} Cy5−anti-TNT antibody for 10 min. The final image shows the resulting increase in fluorescence intensity as the concentration of the OVA−TNP complex increases.

The dose−response curve of the assay is shown in Figure 5B as the net fluorescence intensity versus the concentration of TNP. Each point on the curve is the mean of the six sensing spots in the particular row exposed to the concentration of interest. The data were fitted with a asymmetric sigmoidal giving an equation of fit of $Y = 18959/[1 + (x/6.7)^{-1.55}]$ with $R^2 = 0.998$. The lowest measured concentration, discernible above the mean ± 3SD of the blank, was 5 ng mL^{-1} TNP, although the actual detection limit is probably as low as 1 ng mL^{-1}. The dynamic range of the response was between 5 and 30 ng mL^{-1}; above 30 ng mL^{-1}, the sigmodal dose−response curve reaches a plateau.

CONCLUSIONS

The capability of the array biosensor for measuring the antigen TNT using competitive and, for the first time on a planar surface, displacement assay formats has been demonstrated, along with the measurement of TNT analogues via direct and sandwich immunoassays formats. Each assay format achieved different limits of detection and dynamic ranges. The direct assay obtained the lowest limit of detection, 1 ng mL^{-1} Cy5−DAP−TNP; however, it also had the narrowest dynamic range. The competitive assay had a detection limit of 20 ng mL^{-1} TNT, higher than the equivalent assay run with the fiber-optic biosensor (5 ng mL^{-1}),[24] and it also had the widest dynamic range of all the assay formats investigated, 20−200 ng mL^{-1}. The displacement assay was found to have a limit of detection of 10 ng mL^{-1} TNT, with a dynamic range of 10−50 ng mL^{-1}. The limit of detection for both the competitive and displacement assay formats could be improved by extending the assay time. The lowest concentration detected using the sandwich assay was 5 ng mL^{-1} TNP with a dynamic range of 5−30 ng mL^{-1}.

One of the major advantages of this system is the quick assay times achieved; the direct, competitive, and displacement assays

take 5 min, plus a 7-min regeneration step, while the sandwich assay, due to its two-step nature, takes 15 min. The regeneration step could be decreased by at least 2 min and the assay times could also be shortened, but at the risk of raising the limit of detection. In terms of real-world samples containing unknown concentrations of TNT, the competitive and displacement assays are the only practical formats for detection and quantification, since TNT is not intrinsically fluorescent and free TNT is too small for a sandwich assay.

In addition to offering a choice of assay formats, the other major advantage of the array biosensor over existing technologies is the ability to measure six samples simultaneously on the same waveguide. Increasing the number of channels in the PDMS flow cell could further enhance the number of measured samples. This study has shown the ability of the array biosensor to perform all four types of immunoassays therefore, providing a powerful tool for the detection and quantification of not only TNT but numerous other possible analytes, both large and small, for which there are capture molecules available.

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