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Array biosensor for detection of biohazards

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Abstract

A fluorescence-based biosensor has been developed for simultaneous analysis of multiple samples for multiple biohazardous agents. A patterned array of antibodies immobilized on the surface of a planar waveguide is used to capture antigen present in samples; bound analyte is then quantified by means of fluorescent tracer antibodies. Upon excitation of the fluorophore by a small diode laser, a CCD camera detects the pattern of fluorescent antibody:antigen complexes on the waveguide surface. Image analysis software correlates the position of fluorescent signals with the identity of the analyte. This array biosensor has been used to detect toxins, toxoids, and killed or non-pathogenic (vaccine) strains of pathogenic bacteria. Limits of detection in the mid-ng/ml range (toxins and toxoids) and in the 10^3–10^6 cfu/ml range (bacterial analytes) were achieved with a facile 14-min off-line assay. In addition, a fluidics and imaging system has been developed which allows automated detection of staphylococcal enterotoxin B (SEB) in the low ng/ml range.

Keywords: Array biosensor; Multianalyte detection; Planar waveguides

1. Introduction

A number of investigators have described optical biosensors *capable* of simultaneous analysis of samples for multiple analytes. However, most of the published work describes only detection of a single analyte (Ekins et al., 1990; Ekins and Chu, 1993; Abel et al., 1996; Herron et al., 1997; Blawas et al., 1998; Brecht et al., 1998). The majority of experiments actually demonstrating the simultaneous detection of multiple analytes accomplished this by putting a single sample over multiple, discrete sensing elements (Kakabakos et al., 1992; Parsons et al., 1993; Bakaltcheva et al., 1998; Narang et al., 1998). Berger et al. (1998), on the other hand, utilized several discrete regions of a single sensing surface to monitor four simultaneous reactions on a four-channel surface plasmon resonance system. However, for monitoring complex samples, the label-free methods continue to be susceptible to problems such as low sensitivity and increased backgrounds due to non-specific binding. Wadkins et al. (1997, 1998) and Silzel et al. (1998) avoided such problems by using fluorescent tracer antibodies and performing a measurement insensitive to non-specifically bound proteins (other than the tracer antibody).

The antibody array biosensor described here is composed of three parts: an array of immobilized capture antibodies acting as molecular recognition elements, an image capture and processing system, and an automated fluidics unit (Fig. 1A). Antibodies specific for hazardous analytes are immobilized in discrete regions on an avidin-coated waveguide by flowing solutions of biotinylated antibodies through a network of polymer channels that confine the solutions to separate regions (Ligler et al., 1998a). Unknown sample is subsequently flowed over the substrate in an orientation perpendicular to the stripes of immobilized antibodies and any antigens present in the sample bind to the appropriate analyte-specific loci in the array. Bound antigens are then incubated with a mixture of fluorescently-labeled tracer antibodies. The resultant antibody/antigen/fluorescent-antibody complex is detected using a CCD camera upon excitation by a small diode laser. Auto-

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mated image analysis software correlates the position of the fluorescence with the identity of the hazardous component, with results displayed directly to the user. Each antibody-coated substrate is a fully disposable unit designed to be used repeatedly until one or more analytes are detected.

The central element of the array biosensor is the planar waveguide used to direct evanescent excitation light to fluorophores which are bound (by immune complex) to the waveguide surface. A major problem originally encountered with this sensing element was the stripping of light when a flow cell was attached to the waveguide. To solve this problem, a unique patterned reflective cladding was developed to optically insulate the waveguide from the flow cell (Feldstein et al., 1999). The pattern of this silver-based cladding covers the area where a six-channel flow cell contacts the waveguide. The rest of the waveguide surface is left unclad and is suitable for performing optical immunoassays.

In order to reduce the size and weight of the sensor, the potential for sample carryover, and the possibility of system contamination, a modular fluidics system has been developed (Fig. 1B; Feldstein et al., 1999). This system consists of permanent elements (pump, valves), replaceable subsystems (sample manifold, inlets and outlets), and a disposable unit (waveguide and flow chamber module). Samples do not pass through any valves, thus avoiding the substantial problem of valve clogging by complex or particulate samples. Further, the system has been designed to include an easily replaceable sample manifold in order to eliminate the problem of cross-contamination from sequential introduction of samples. The valves and pump are computer controlled to operate the assay protocols in an automated fashion. A unique feature of the fluidics system is the ability to assay up to six samples simultaneously using a single multi-channel pump and a single switching valve.

Fluorescent images are analyzed using data acquisition software developed at NRL (Feldstein et al., 1999). The signal from each antigen-specific spot is automatically corrected for non-specific binding of the fluorescent reagent and for the slight variations in excitation intensity across the surface of the waveguide.

To date, work performed using the array biosensor has demonstrated that mixtures of fluorescent tracer antibodies can be used in rapid assays for protein, bacterial, and viral analytes with sensitivities similar to standard ELISAs (Fig. 2; Wadkins et al., 1998; Rowe et al., 1999a,b). Results obtained using mixtures of antibodies were not significantly different from those obtained from parallel assays utilizing individual tracers (Rowe et al., 1999b). Furthermore, mixtures of analytes could also be detected and identified. Moreover, analytes present in complex sample matrices such as blood and urine could be detected and quantified using the automated data analysis program, provided a suitable 'clean' sample was assayed on the same slide (Rowe et al., 1999a).

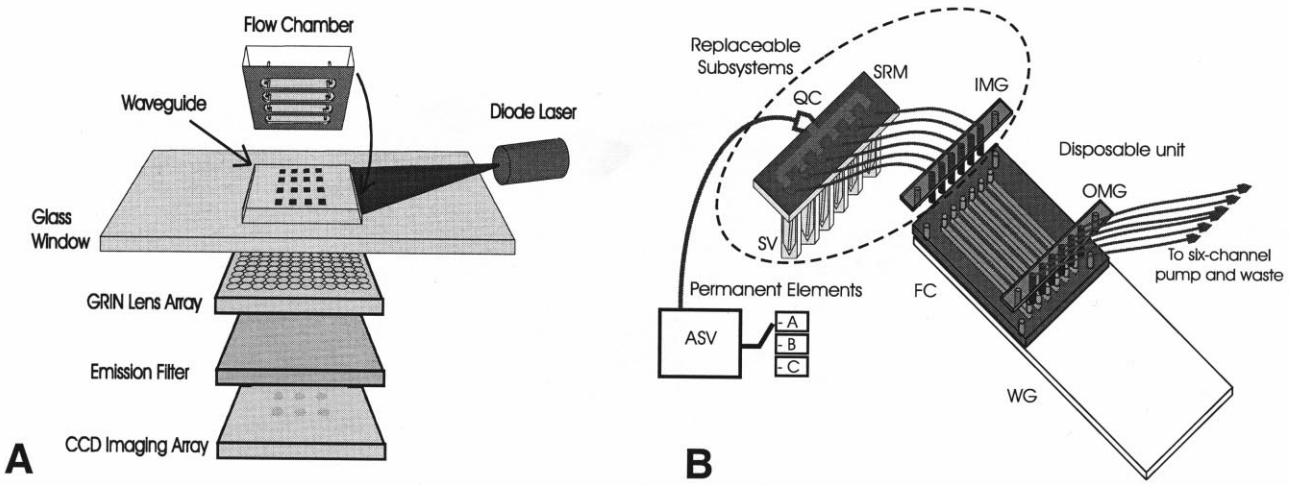
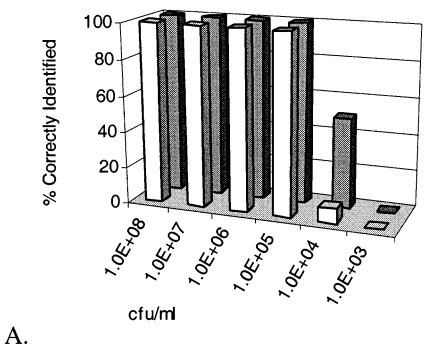
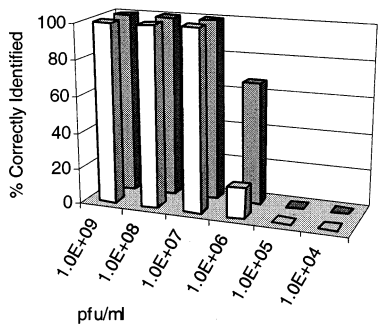


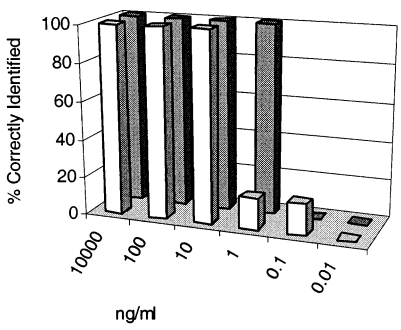
Fig. 1. Optics and fluidics components of the array sensor. (A) Optical components. The patterned waveguide is placed in a mounting scaffold for reproducible alignment. Fluorescent complexes on the waveguide surface are evanescently excited by a 635 nm diode laser. A 2-dimensional array of GRIN lenses focuses the pattern of fluorescence onto a CCD imaging array; filters have been installed to reject stray excitation and scattered light. The image of the fluorescent pattern is then captured in digital format and can be analyzed using an automated data analysis program. (B) Fluidics components. The fluidics system consists of permanent elements, replaceable subsystems, and a disposable unit (Feldstein et al., 1999). The permanent elements include an automated switching valve (ASV) that selects either air (A), buffer (B), or fluorescently-labeled antibodies (C), and an output manifold with a gasket layer (OMG) to direct fluids to a six-channel peristaltic pump (not shown). The replaceable subsystem consists of a sample–reagent manifold (SRM) that draws samples from the sample vials (SV) or reagents, as selected at the switching valve; quick connectors (QC) allow rapid and easy replacement of the sample subsystem. An input manifold employing a gasket layer (IMG) is used to direct the output of the sample–reagent manifold into the disposable unit consisting of a multi-channel flow cell (FC) permanently attached to a waveguide (WG).



A.



B.



C.

Fig. 2. Comparison of array biosensor results with ELISA results (Rowe et al., 1999b). The array sensor was used to test 126 blind samples containing either a bacterial, a viral, or a protein analyte. Analyte concentrations ranged from six to seven orders of magnitude; six blind samples were analyzed at each concentration. The ordinate indicates the number of samples at each concentration that were correctly detected and identified (expressed as a percentage of the total). The abscissa indicates the analyte concentration in each set of six samples. White bars are indicative of results obtained with the array biosensor; ELISA results are indicated in gray. (A) Detection of bacterial analyte, *B. globigii*. (B) Detection of viral analyte, MS2. (C) Detection of protein analyte, SEB.

These previous experiments were performed using a non-automated version of the array biosensor, which requires user manipulation of samples, flow chamber modules, and optical waveguides. While the strength of this sensor is its ability to detect multiple analytes simultaneously (Wadkins et al., 1998; Ligler et al., 1998a,b; Rowe et al., 1999a,b), the purpose of this report is to document further development of rapid assays for potentially hazardous analytes at concentrations similar to competing technologies. In addition, we describe in Section 3.2 testing of a fully automated system utilizing a computer-controlled fluidics system (Fig. 1), the data analysis program, and flow guides which have been permanently mounted onto patterned waveguides. This demonstration of full automation is the first description of an array sensor which requires no user intervention after samples are loaded.

2. Materials and methods

2.1. Antibodies and analytes

Antibodies and the majority of antigens used in this work were generous gifts of Woody Johnson and Jennifer Aldrich at Naval Medical Research Center (NMRC, Bethesda, MD). The NMRC antibody preparations were provided after purification with Protein A or Protein G chromatography. The anti-*Bacillus anthracis* antibody had also been affinity purified by J. Aldrich and W. Johnson. Individual components in the array assays and their sources are listed in Table 1.

None of the bacterial analytes used in these studies were pathogenic. The *B. anthracis* and *Francisella tularensis* antigens were non-pathogenic vaccine strains. *Brucella abortus* antigen was supplied as a formalin-killed stock of the ATCC type strain for Biotype 1; these stocks were tested for viability prior to shipping. Botulinum toxoids A and B were prepared by formaldehyde inactivation of the toxins and were tested for residual toxicity by injection into mice prior to shipment to NRL. All solutions, glassware, etc. containing bacteria or toxic analytes (SEB, ricin, and cholera toxin) were handled by personnel wearing gloves and appropriate personal protective gear (lab coat, safety goggles). All equipment, benchtops, instruments, etc. exposed to these solutions were disinfected with a 20% bleach solution and were rinsed with distilled water. Analyte solutions were also treated with bleach (20% final concentration) before disposal. Contaminated disposables (test tubes, pipette tips, used waveguides) were placed in biohazard containers and later incinerated.

2.2. Preparation of capture and detection antibodies

All biosensor assays utilized a standard sandwich immunoassay format. Antibodies used as capture reagents were labeled with biotin for immobilization in patterns on NeutrAvidin-coated waveguides. Antibody preparations were biotinylated using a 5-fold molar excess of biotin-LC-NHS ester (Pierce, Rockford, IL), according to previously published protocols (Rowe et

al., 1999b). Unincorporated biotin was separated from labeled protein by gel filtration on BioGel P-10.

Fluorescent tracer antibodies were labeled with the cyanine dye, Cy5 Bisfunctional Reactive Dye ($\lambda_{ex} = 649$ nm, $\lambda_{em} = 670$ nm; Amersham Life Science Products, Arlington Heights, IL), according to previously published methods (Rowe et al., 1999a); unincorporated dye was separated from labeled protein by chromatography on BioGel P-10.

2.3. Preparation of waveguides

Two cleaning methods were utilized to ensure uniform immobilization of NeutrAvidin onto microscope slide surfaces. Conventional glass microscope slides (DaiggerBrand, Daigger, Wheeling, IL) were cleaned using a combination of MeOH:HCl (1:1) and concentrated sulfuric acid rinses, according to published protocols (Cras et al., 1999). Glass slides with protected silver cladding (Feldstein et al., 1999) were purchased from Opticoat Associates, Inc. (Chelmsford, MA). Due to delamination of the silver layer in the MeOH:HCl wash, the silver-cladded slides were instead cleaned in 10% KOH in isopropanol (Cras et al., 1999). Following the appropriate cleaning procedures, the glass and silver-coated slides were rinsed exhaustively in deionized water and were dried under a stream of nitrogen.

NeutrAvidin Biotin Binding Protein (Pierce) was covalently immobilized onto the glass and silver-coated waveguides essentially according to published procedures (Bhatia et al., 1989; Ligler et al., 1991; Feldstein et al., 1999; Rowe et al., 1999a,b). Mercaptopropyltriethoxy silane was used in place of the trimethoxy derivative, due to its lower toxicity.

Biotin-labeled capture antibodies were patterned onto the NeutrAvidin-coated waveguides using polydimethyl siloxane (PDMS) patterning guides as described (Ligler et al., 1998a,b) The slides were patterned by overnight incubation with 20 μg/ml biotinylated goat anti-B. abortus IgG or 10 μg/ml of the other biotinylated antibodies in 10 mM Na phosphate pH 7.4/10 mM NaCl. After rinsing in phosphate-buffered saline containing 0.05% Tween 20 (PBST), the slides were stored in 10 mM Na phosphate pH 7.5 until use. Alternatively, the rinsed, patterned slides were incubated in 10 mM Na phosphate pH 7.4 containing 10 mg/ml bovine serum albumin (BSA) and dried under a stream of nitrogen.

Permanent flow cells were attached to slides to be assayed using the automated fluidics system. Black polymethylmethacrylate (PMMA) flow cells (High Tech Machining, Bradenton, FL) were clamped onto the patterned surface of the dried waveguides using small binder clamps. Epoxy (EP30, Master Bond, Inc., Hackensack, NJ) was allowed to wick in from each end and eventually filled all zones of contact between the slide and the flow guide. The slides were left clamped for 2 days while the epoxy cured and could then be used for automated assays.

Table 1
Sources of antigens and antibodies used in this study

Analyte	Source	Antibody	Source
Bacterial analytes			
B. anthracis Sterne[a]	NMRC[b]	Rabbit polyclonal IgG	NMRC
F. tularensis LVS[c]	NMRC	Goat polyclonal IgG	
		Monoclonal FT-03-A-3 (detection only)	NMRC
B. abortus (ATCC 23448)[d]	NMRC	Goat polyclonal IgG	NMRC
Toxins			
S. aureus enterotoxin B (SEB)	Toxin technology[e]	Rabbit polyclonal IgG	NMRC
Botulinum toxoids A and B[f]	NMRC/U. Wisc.	Goat polyclonal IgG	NMRC
Cholera toxin	Calbiochem[g]	Rabbit polyclonal IgG	Biogenesis[h]
Ricin	Sigma[i]	Goat polyclonal IgG	NMRC
		Monoclonal RIC-03-A-G1 (detection only)	NMRC

[a] FDA-approved vaccine strain; whole cell preparation.
[b] Bethesda, MD.
[c] Vaccine strain; whole cell preparation.
[d] Formalin-inactivated. The concentration (2.8×10^9 cfu/mg) was determined prior to inactivation. Killed material was tested for residual viability by plating undiluted stock onto blood agar and observation for 4 days.
[e] Sarasota, FL.
[f] Original source of toxoids was University of Wisconsin, Madison Food Research Institute. Toxins were inactivated by dialysis in 0.1 M Na phosphate, 0.05 M NaCl, and 0.5% formaldehyde (pH 7.4) for 7 days with daily buffer changes. No residual toxicity was observed up to 96 h after injection of 50 μg into live mice.
[g] La Jolla, CA.
[h] Brentwood, NH.
[i] St. Louis, MO.

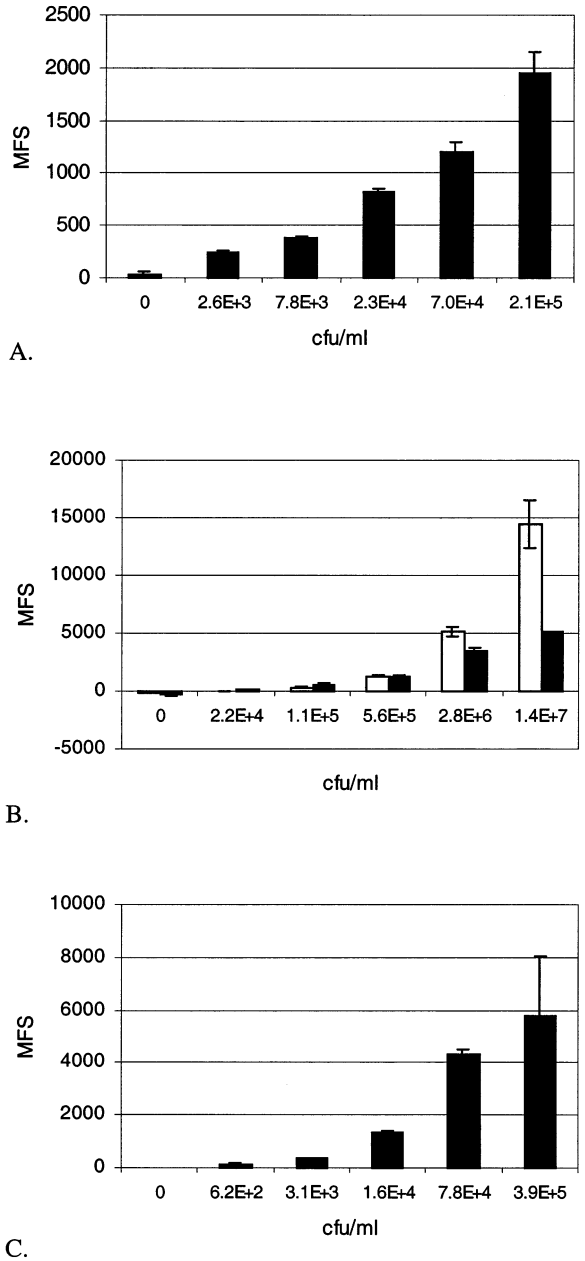


Fig. 3. Dose–response curves for bacterial analytes. Slides were patterned with polyclonal goat or rabbit IgG directed against the appropriate analyte. Off-line assays were performed as described in Materials and methods, Section 2.6.1. Shown are mean fluorescence signals (MFS, mean intensities of all antigen-specific spots with local background values subtracted) for each analyte as a function of analyte concentration. (A) Formalin-killed *B. abortus*. Goat polyclonal antibodies (20 μg/ml) were used for both capture and detection. (B) *F. tularensis* LVS vaccine strain. Goat antibodies were used for capture (10 μg/ml). Assays performed using monoclonal tracer antibodies (FT-03-A-G3, 10 μg/ml) are indicated with open bars; closed bars indicate goat polyclonal tracer antibodies (10 μg/ml). (C) *B. anthracis* Sterne. Rabbit polyclonal antibodies (10 μg/ml) were used for both capture and detection. Error bars indicate SEM ($n = 6$).

2.4. Optical components

Optical components of the array sensor are described in greater details elsewhere (Fig. 1A; Feldstein et al., 1999). Briefly, the immunosensor array was interrogated using 635 nm diode laser coupled into the distal end of the waveguide. To achieve uniform excitation of the patterned sensing region, a line generator and cylindrical lens were utilized. The evanescently excited pattern of fluorescence was focused through a 2-dimensional array of graded index lenses (Golden, 1998) onto a large-area, peltier cooled CCD camera.

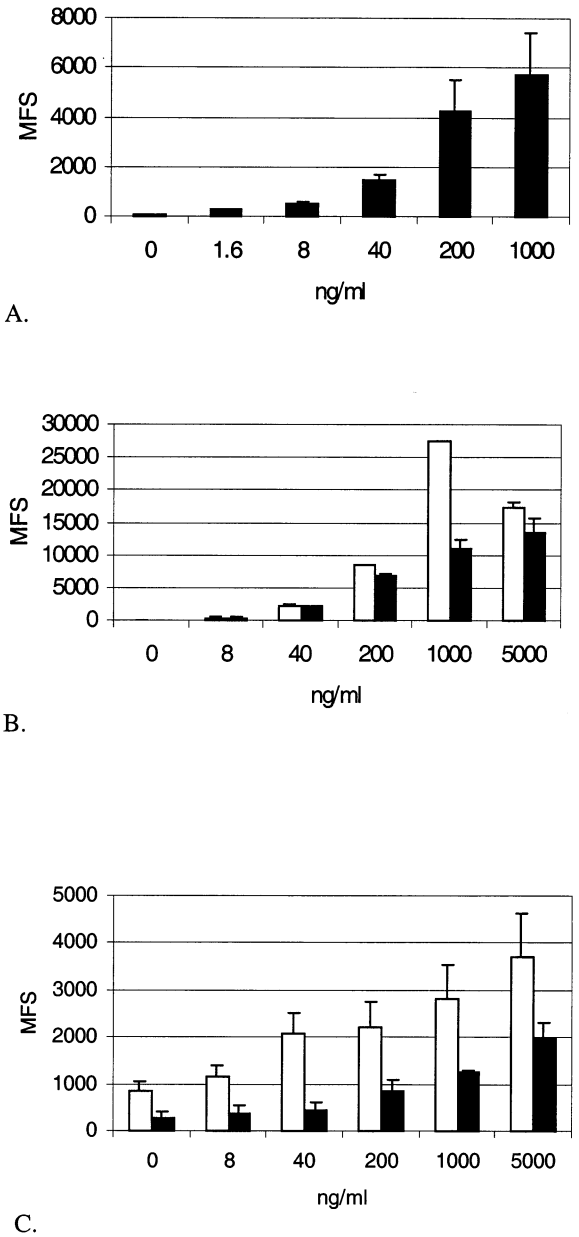


Fig. 4. Dose–response curves for toxins. Slides were patterned with 10 μg/ml appropriate polyclonal goat IgGs and assays were performed as described in Materials nad methods, Section 2.6.1, using 10 μg/ml of tracer antibody. Shown are mean fluorescence signals (MFS) for each analyte as a function of analyte concentration. (A) Cholera toxin. (B) Ricin. Open bars indicate that monoclonal RIC-03-A-G1 was used as tracer; closed bars indicate that goat polyclonal tracer. (C) Botulinum toxoids. Open bars indicate toxoid A; closed bars indicate toxoid B. Error bars indicate SEM ($n = 6$).

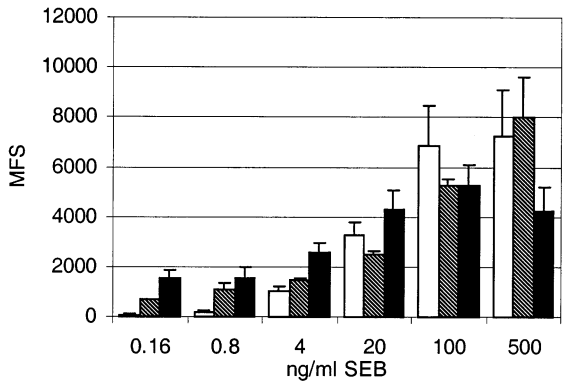


Fig. 5. Comparison of results obtained with off-line and on-line assays. Samples containing SEB were analyzed using the 14-min off-line assays described in Materilas and methods, Section 2.6.1, or the 20-min on-line assay described in Section 2.6.2. Off-line assays performed using patterned glass slides are indicated with open bars; the same assays performed using silver-cladded slides are indicated with striped bars. Black bars indicate automated, on-line assays (see Section 2.6.2) performed using silver-cladded slides. Shown are mean fluorescence signals (MFS) as a function of analyte concentration. Error bars indicate SEM ($n = 6$).

Longpass (665 nm) and bandpass (670 nm) filters were installed to reject excitation and scattered light.

2.5. Automated fluidics components

The automated fluidics system has been described in greater detail elsewhere (Feldstein et al., 1999) and is represented in Fig. 1B. Briefly, the patterned waveguide with permanently mounted flow cell was attached to the fluidics system via two press-in inlet and outlet manifolds. These manifolds were fitted with Neoprene gaskets to ensure a fluid-tight seals. The outlet manifold was connected to a six-channel peristaltic pump. The inlet manifold attached to a sample manifold containing six air-tight sample vials. The sample manifold was connected to a modular multi-position valve which controlled the composition of solutions pumped through the sample vials and subsequently through the flow cell.

2.6. Assays

2.6.1. Off-line assays

Off-line assays were performed as previously described (Rowe et al., 1999a), except as indicated. Analyte concentrations were chosen to reflect the current sensitivities of the array sensor and competing sensor technologies. Patterned slides were placed in contact with PDMS assay flow guides, such that the flow channels were perpendicularly oriented to the stripes of capture antibody. Assays were performed under flow, using the following protocol. The concentrations of tracer reagents were 20 μg/ml for *B. abortus* antibodies and 10 μg/ml for all others used.

Step I. 1.2 min wash with PBST containing 1 mg/ml BSA (PBSTB, 1 ml); flow rate, 0.8 ml/min
Step II. 7 min incubation with sample (0.8 ml); flow rate, 0.11 ml/min
Step III. 1.2 min rinse with PBSTB (1 ml); flow rate, 0.8 ml/min
Step IV. 3 min incubation with Cy5-labeled tracer antibody (0.4 ml); flow rate, 0.133 ml/min
Step V. 1.2 min rinse with PBSTB (1 ml); flow rate, 0.8 ml/min

After removal of the PDMS flow guides from the slides, the slides were rinsed with deionized water, were dried under a stream of nitrogen, and were imaged using the CCD.

2.6.2. On-line assays

Automated on-line assays were performed after permanent attachment of PMMA flow cells to patterned, dried slides (Fig. 1B). Samples (1 ml) were pipetted into the sample vials, which were then screwed into the sample manifold. An inlet or outlet manifold was attached to each end of the flow cell and tightened such that the connection was fluid-tight. Using the pumping and valving apparatus described in Section 2.5, above, the assays were performed under flow, using the following protocol.

Step I. 10 min incubation with sample (1 ml); flow rate, 0.1 ml/min
Step II. 2.5 min rinse with PBSTB (1.5 ml); flow rate, 0.6 ml/min
Step III. 4 min incubation with 10 μg/ml Cy5-labeled rabbit anti-SEB (0.4 ml); flow rate, 0.1 ml/min
Step IV. 2.5 min rinse with PBSTB (1.5 ml); flow rate, 0.6 ml/min

After the final rinse, the channels in the flow cell were emptied of buffer and the slide was imaged using the CCD.

2.7. Calculations

Fluorescence intensity data were extracted from the CCD images using an automated data analysis program (Feldstein et al., 1999). This program provided the mean fluorescence intensity for each antigen-specific spot, localized background intensities and associated noise, signal-to-noise ratios, and background-subtracted mean fluorescent signals (MFS) for each spot in tabular format. MFS were calculated by subtracting the mean local background fluorescence (taken on each side of the fluorescent spot) from the mean fluorescence within the spot. This correction for non-specific binding assumes that the degree of binding in the antibody-coated and non-coated regions is the same. These values are presented in Figs. 3–5.

Limits of detection were determined to be the lowest concentration tested at which the mean antigen-specific MFS was greater than the negative control values (buffer used in place of sample) *plus* three standard errors of means (SEM).

3. Results and discussion

3.1. Off-line, non-automated assays

Assays were developed for six analytes potentially capable of causing illness or disease. These assays consisted of simple sandwich immunoassays performed on the surface of planar waveguides using biotinylated capture antibodies for antigen recognition; these platforms were prepared in advance of the assays and incubation of sample with the prepared substrates required only 7 min. Bound antigens were then detected by a 3 min incubation with fluorescent tracer molecules. Following this 14-min assay procedure, the location and intensity of evanescently excited fluorescent sandwiches were determined using a CCD and an automated analysis program to extract data from captured images. The total time required to perform the biochemical assay and extract data was less than 20 min.

3.1.1. Detection of bacteria

Dose–response curves for the three bacterial analytes are shown in Fig. 3. The limit of detection obtained for *B. abortus* (Panel A) was 2.6×10^3 cfu/ml. Lower concentrations of *B. abortus* often gave fluorescent signals which were visible to the eye, but which were not always three SEMs above background or negative control values. A monoclonal antibody directed against *B. abortus* (MEL-03-A-A, NMRC) was also tested as a tracer reagent and failed to give fluorescent signals significantly above background values at analyte concentrations of 10^5 cfu/ml or less.

In contrast to the negative results obtained using monoclonal antibodies for detection of *B. abortus*, monoclonal antibodies performed better than the polyclonal IgG preparation in *F. tularensis* assays. Panel B shows the dose–response curves obtained when monoclonal antibody (white bars) and polyclonal IgGs (closed bars) were used as tracer reagents. Goat polyclonal antibodies were utilized as the immobilized capture reagents in both experiments. Although use of anti-*F. tularensis* monoclonal (clone no. FT-03-A-3) as tracer resulted in a higher MFS than the polyclonal tracer, limits of detection for both were 1.1×10^5 cfu/ml.

A dose–response curve was also determined for the vaccine strain of *B. anthracis* (Panel C). The calculated limit of detection was 624 cfu/ml, the lowest concentration of *B. anthracis* tested. More recent results indicate a higher limit of detection when a non-affinity-purified antibody preparation is used.

3.1.2. Detection of toxins and toxoids

Assays were developed for cholera toxin, ricin, and botulinum toxoids A and B (Fig. 4). A dose–response curve for cholera toxin was determined using rabbit polyclonal antibodies for both capture and detection (Panel A). The limit of detection was calculated to be 1.6 ng/ml, the lowest concentration tested. Non-specific binding of fluorescent antibody was not observed in negative controls.

Panel B shows the dose–response curves for ricin using monoclonal antibodies (open bars) and polyclonal antibodies (closed bars) as tracers. Although the MFS values did not significantly differ at ricin concentrations in the ng/ml range, the MFS obtained at high ricin concentrations were significantly greater in assays where the monoclonal tracer antibodies were used compared to those which were obtained with polyclonal tracers. Limits of detection for assays for both tracers were 8 ng/ml. This was the lowest concentration tested using the array sensor.

Panel C shows the dose–response curves for botulinum toxoids A (open bars) and B (closed bars); limits of detection were calculated to be 40 ng/ml for toxoid A and 200 ng/ml for toxoid B. In contrast to the assays for cholera toxin and ricin, non-specific binding was observed in all negative controls (PBSTB applied in place of sample). The non-specific binding of fluorescent antibody accounted for the high MFS in negative controls in assays for both toxoids. A mouse monoclonal (BOT-01-B-G1) directed against botulinum toxin was also tested for efficacy in the array sensor (data not shown), but as in the case of the *B. abortus* antibodies, the polyclonal antibody (shown) was superior to the monoclonal in both ELISA and array formats.

3.2. On-line, automated assays

Staphylococcal enterotoxin B (SEB) was the standard analyte used to test the efficacy of the automated fluidics module. SEB was assayed using several different formats (Fig. 5). Samples containing varying concentrations of SEB were assayed using the standard off-line sandwich 14-min immunoassay utilized above. Off-line assays were performed using either patterned glass slides (open bars) or silver-cladded slides (striped bars) which had been subjected to identical conditions of avidin immobilization and patterning of capture antibodies. These off-line assays were used to assess the effect of the reflective cladding on evanescent excitation, background scattering, etc. Assays were also run on-line, using the automated fluidics system (closed bars), to test the efficacy and reproducibility of the automated system.

Although the signals in the silver-clad slides were higher at low SEB concentrations, this difference was not significant ($P < 0.05$). In addition, there was no significant difference between any of the MFS values from the three assays at the higher SEB concentrations. However, the limit of detection for the on-line assay (black bars, 20 ng/ml SEB) was higher than the limits of detection for the two off-line assays (white, striped bars); both off-line assays had limits of detection of 4 ng/ml. This difference was due to the significantly higher *variability* in the background values of the on-line assays, as well as higher localized background values. This variability may potentially be due to differences in excitation caused by the presence of the black PMMA flow cell or by the presence of epoxy within the flow channels. It was observed that, in cases where the optical epoxy used to attach the flow cells to the waveguide had seeped into the flow channels, the epoxy bound the fluorescently-labeled tracer antibodies (data not shown).

Other methods have been used to test for the above analytes, several of which are discussed elsewhere in this issue. These methods include light addressable potentiometric sensors (LAPS), of which the Threshold System™ is the most well known (Thompson and Lee, 1992; Chambers and Valdes, 1992; Lee et al., 1993; Menking and Goode, 1993; Dill et al., 1994a,b; Lee et al., 1995; Menking et al., 1995), piezoelectric sensors (Carter et al., 1995a,b; Harteveld et al., 1997), biorefractometric sensors (Bioarski et al., 1994), impedance-based sensors (DeSilva et al., 1995), and evanescent wave fiber optic sensors (Ogert et al., 1992; Menking and Goode, 1993; Kumar et al., 1994; Ogert et al., 1994; Wijesuria et al., 1994; Tempelman et al., 1996; King et al., 1999). In general, limits of detection were in the pg/ml to low ng/ml range for toxins and in the 10^3–10^4 cfu/ml range for bacterial analytes, which are approximately the same as those obtained using the array sensor. Picogram detection limits for toxins have been obtained using LAPS (Thompson and Lee, 1992; Chambers and Valdes, 1992; Dill et al., 1994a,b); however, LAPS assays are generally not considered to be rapid detection methods, typically requiring at least 1 h to complete, even when antibody:antigen incubations have been shortened considerably. Moreover, sensitivity in the LAPS system is dependent on the sample volume used.

Like other evanescent wave techniques, the array sensor has the advantage of rapid response time and relative insensitivity to complex sample matrices (Rowe et al., 1999a). Matrix effects are a common problem encountered with methods based on refractive index changes (surface plasmon resonance, biorefractometry). The fact that only labeled molecules generate a fluorescent signal in the current biosensor enables the discrimination of tracer binding from non-specific adsorption of sample components to the sensing surface. Furthermore, in evanescent wave sensors, the narrow penetration depth of the evanescent field, typically 100–200 nm, allows monitoring of binding events on the surface of the waveguide with little interference from the bulk solution. Hence, real-time measurements can be made on samples which are turbid or contain naturally occurring fluorescent particles. The array sensor has an additional advantage in its ability to analyze multiple samples at the same time: this allows controls and standards of known concentrations to be analyzed simultaneously with the sample of interest. Thus, quantitation can be carried out more rapidly than in methods where standards must be analyzed separately from the unknown sample (Chambers and Valdes, 1992; Ogert et al., 1992; Menking and Goode, 1993; Bioarski et al., 1994; Ogert et al., 1994; DeSilva et al., 1995; Tempelman et al., 1996).

A possible application of the array sensor is routine monitoring of environmental samples. The sandwich immunoassay format described here is designed to allow reuse of sensor substrates when negative results are obtained; competitive immunoassays require regeneration of substrates and removal of bound analyte before subsequent analyses. Although reuse of *array* substrates has not yet been demonstrated, optical fibers used in evanescent wave assays (using analogous surface chemistries and assay formats) have been used for up to 12 assays (Shriver-Lake et al., 1992; Ligler et al., 1993, 1998b) without losing the ability to detect analyte in positive samples.

The key strength of the array sensor is its ability to analyze multiple samples for multiple analytes simultaneously. Only planar waveguide systems have demonstrated the ability to detect multiple analytes on a single sensor substrate (Pritchard et al., 1995; Plowman et al., 1999; Silzel et al., 1998). Moreover, the array sensor has demonstrated the ability to detect multiple classes of analytes simultaneously (Rowe et al., 1999b) in a rapid assay format. In its current configuration, the array sensor is capable of running 36 assays simultaneously.

The array assays previously described (Rowe et al., 1999a,b; Wadkins et al., 1997, 1998) and those developed for the hazardous analytes described above require multiple manipulations. However, the array sensor is in the process of being made smaller, more portable, and totally automated. The automated fluidics protocol significantly streamlines the manipulations involved with running the assays; the user simply adds sample to appropriate vials, attaches buffer and reagent reservoirs, attaches the input and output manifolds to the pre-patterned waveguide, and starts the pre-programmed protocol. Following the assay, the automated data analysis program extracts data from the imaged slide and presents it to the user in both a tabular format and in an easy-to-read bubble-chart,

clearly indicating the absence or presence of analyte in each sample lane. Although the data shown here indicate a 5-fold loss in sensitivity in the fully automated assays, this is primarily due to increased variability in the background, which may be correctable when the process of permanently attaching the flow guides has been optimized.

4. Conclusion

The array biosensor assays multiple samples simultaneously for multiple analytes. Not only does it exhibit sensitivity comparable with other antibody-based methods that require sample aliquots to be assayed individually for different agents, but the multianalyte assays have been automated. The off-line assays took an average of 15–18 min to complete the biochemical assays, image the slide, and collect the data. Limits of detection for bacterial analytes ranged from 624 cfu/ml (B. anthracis) to 10^5 cfu/ml for *F. tularensis*: sensitivity was highly dependent on the antibody preparations used. Sensitivity for toxins also varied according to antibodies used for detection but low ng/ml concentrations of toxins were generally detected. The total assay time was increased to 20 min when the automated fluidics and imaging systems were utilized. Although the limit of detection was increased 5-fold when using the automated system, the MFS were not significantly different when comparing the results obtained using the automated assays and those run off-line. Operator manipulations were greatly simplified in the automated assays compared to the off-line version, requiring only that the operator pipette sample and tracer antibody into specific tubes before starting the assays. The sample introduction procedure could also be automated for continuous monitoring applications.

The array biosensor is in the laboratory breadboard stage and needs to be converted to a manufacturable, fieldable system prior to deployment. Yet the current system has demonstrated sensitivity and specificity for toxins and pathogenic bacteria in rapid assays and the capability of assaying for multiple analytes simultaneously. It has been designed as a portable instrument with optical, fluidic, and sensing components amenable to miniaturization. The major hurdles left to address are the further miniaturization of the fluidic and optical components: this work is well underway in our laboratory. However, as far as the assays are concerned, the design of the sensing surface and optics is amenable to incorporation of an ever increasing number of assays on each waveguide.

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References

Abel, A.P., Weller, M.G., Duveneck, G.L., Ehrat, M., Widmer, H.M., 1996. Fiber-optic evanescent wave biosensor for the detection of oligonucleotides. Anal. Chem. 68, 2905–2912.

Bakaltcheva, I.B., Shriver-Lake, L.C., Ligler, F.S., 1998. A fiber optic biosensor for multi-analyte detection: Importance of preventing fluorophore aggregation. Sens. Actuators B. 51, 46–51.

Berger, C.E.H., Beumer, T.A.M., Kooyman, R.P.H., Greve, J., 1998. Surface plasmon resonance multisensing. Anal. Chem. 70, 703–706.

Bhatia, S.K., Shriver-Lake, L.C., Prior, K.J., Georger, J.H., Calvert, J.M., Bredehorst, R., Ligler, F.S., 1989. Use of thiol-terminal silanes and heterobifunctional crosslinkers for immobilization of antibodies on silica surfaces. Anal. Biochem. 178, 408–413.

Bioarski, A., Bhullar, G., Miller, L., Zulich, A., Burans, J. 1994. Detection of toxins and biological agents using a biorefractometer. In: Berg, D.A., Williams, J.D. Jr., Reeves, P.J. (Eds.), Proc. 1993 ERDEC Scientific Conference on Chemical Defense Research, 16–19 November 1993, Report No. ERDEC-SP-024 August 1994, pp. 51–60.

Blawas, A.S., Oliver, T.F., Pirrung, M.C., Reichert, W.M., 1998. Step-and-repeat photopatterning of protein features using caged-biotin-BSA: characterization and resolution. Langmuir 14, 4243–4250.

Brecht, A., Klotz, A., Barzen, C., Gauglitz, G., Harris, R.D., Quigley, G.R., Wilkinson, J.S., Sztajnbok, P., Abuknesha, R., Gascón, J., Oubiña, A., Barceló, D., 1998. Optical immunoprobe development for multiresidue monitoring in water. Anal. Chim. Acta 362, 69–79.

Carter, R.M., Mekalanos, J.J., Jacobs, M.B., Lubrano, G.J., Guibault, G.G., 1995a. Quartz crystal microbalance detection of Vibrio cholerae O139 serotype. J. Immunol. Methods 187, 121–125.

Carter, R.M., Jacobs, M.B., Lubrano, G.J., Guibault, G.G., 1995b. Piezoelectric detection of ricin and affinity purified goat anti-ricin antibody. Anal. Lett. 28, 1379–1386.

Chambers, J.P., Valdes, J.J., 1992. Detection of picogram quantities of botulinus toxin B using the light addressable potentiometric sensor. Edgewood Research Development and Engineering Center, ERDEC Report No. DAAA15-89-C-0008.

Cras, J.J., Rowe, C.A., Nivens, D.A., Ligler, F.S., 1999. Comparison of chemical cleaning methods of glass in preparation for silanization, Biosens. Bioelectron. 14, 683–688.

DeSilva, M.S., Zhang, Y., Hesketh, P.J., Maclay, G.J., Gencel, S.M., Stetter, J.R., 1995. Impedance based sensing of the specific binding reaction between *Staphylococcus* enterotoxin B and its antibody on an ultra-thin platinum film. Biosens. Bioelectron. 10, 675–682.

Dill, K., Blomdahl, J.A., Lydon, S.R., Olson, J.D. 1994. Improvements in Threshold immunoassay technology. In: Berg, D.A., Williams, J.D. Jr., Reeves, P.J. (Eds.), Proc. 1993 ERDEC Scientific Conference on Chemical Defense Research, 16–19 November 1993, Report No. ERDEC-SP-024 August 1994, pp. 45–50.

Dill, K., Lin, M., Poteras, C., Fraser, C., Hafeman, D.G., Owicki, J.C., Olson, J.D., 1994b. Antibody-antigen binding constants determined in solution-phase with threshold membrane-capture system: binding constants for anti-fluorescein, anti-saxitoxin, and anti-ricin antibodies. Anal. Biochem. 217, 128–138.

Ekins, R., Chu, R., 1993. Multianalyte testing. Clin. Chem. 39, 369–370.

Ekins, R., Chu, F., Biggart, E., 1990. Fluorescence spectroscopy and its application to a new generation of high sensitivity, multi-microspot, multianalyte, immunoassay. Clin. Chim. Acta 194, 91–114.

Feldstein, M.J., Golden, J.P., Rowe, C.A., MacCraith, B.D., Ligler, F.S., 1999. Array biosensor: optical and fluidics systems. J. Biomed Microdevices 1, 139–153.

Golden, J.P., 1998. Chemical sensor using two-dimensional lens array. US Patent. No. 827 5, 748.

Harteveld, J.L.N., Nieuwenhuizen, M.S., Wils, E.R.J., 1997. Detection of staphylococcal enterotoxin B employing a piezoelectric crystal immunosensor. Biosens. Bioelectron. 12, 661–667.

Herron, J.N., Christensen, D.A., Wang, H.-K., Caldwell, K.D., Janatova, V., Huang, S.-C., 1997. Apparatus and methods for multianalyte homogeneous fluoroimmunoassays. US Patent. No. 677 5, 196.

Kakabakos, S.E., Christopoulos, T.K., Diamandis, E.P., 1992. Multianalyte immunoassay based on spatially distinct fluorescent areas quantified by laser-excited solid-phase time-resolved fluorometry. Clin. Chem. 38, 338–342.

King, K.D., Anderson, G.P., Bullock, K.E., Regina, M.J., Saaski, E.W., Ligler, F.S., 1999. Detecting staphylococcal enterotoxin B using an automated fiber optic biosensor. Biosens. Bioelectron. 14, 163–170.

Kumar, P., Colston, J.T., Chambers, J.P., Rael, E.D., Valdes, J.J., 1994. Detection of botulinum toxin using an evanescent wave immunosensor. Biosens. Bioelectron. 9, 57–63.

Lee, W.E., Jacobson, T.V., Thompson, H.G., 1993. Characteristics of the biochemical detector sensor. Defence Research Establishment Suffield. Can. Suffield Memorandum 1407, 1–23.

Lee, W.E., Hall, J.G., Thompson, H.G., 1995. Fluorescence immunofiltration assay of *Brucella melitensis*. Defence Research Establishment Suffield. Can. Suffield Memorandum, 1402.

Ligler, F.S., Calvert, J., Georger, J., Shriver-Lake, L.S., Bhatia, S., 1991. Immobilization of active agents on substrates with a silane and heterobifunctional. Crosslinker. US Patent. No. 077 5, 210.

Ligler, F.S., Golden, J.P., Shriver-Lake, L.C., Ogert, R.A., Wijesuria, D., Anderson, G.P., 1993. Fiber-optic biosensor for detection of hazardous materials. Immunomethods 3, 122–127.

Ligler, F.S., Conrad, D.W., Golden, J.P., Feldstein, M.J., MacCraith, B.D., Balderson, S.D., Czarnaski, J., Rowe, C.A., 1998a. Array biosensor for multi-analyte sensing. SPIE 3258, 50–55.

Ligler, F.S., Anderson, G.P., Davidson, P.T., Foch, R.J., Ives, J.T., King, K.D., Page, G., Stenger, D.A., Whelan, J.P., 1998b. Remote sensing using an airborne biosensor. Environ. Sci. Technol. 32, 2461–2466.

Menking, D.G., Goode, M.T., 1993. Evaluation of cocktailed antibodies for toxin and pathogen assays on the light addressable potentiometric sensor. In: Williams, J.D., Berg, D.A., Reeves, D.A. (Eds.), Proc. 1992 ERDEC Scientific Conference of Chemical Defense Research, 17–20 November 1992, Report No. ERDEC-SP-007. June 1993, pp. 103–109.

Menking, D.G., Thompson, R.G., Heitz, J.M., Anis, N.A., 1995. Antibody-based detection of toxins of biological origin. Edgewood Research Development and Engineering Center, Report No. ERDEC-TR-279.

Narang, U., Gauger, P.R., Kusterbeck, A.W., Ligler, F.S., 1998. Multianalyte detection using a capillary-based flow immunosensor. Anal. Biochem. 255, 13–19.

Ogert, R.A., Brown, J.E., Singh, B.R., Shriver-Lake, L.C., Ligler, F.S., 1992. Detection of *Clostridium botulinum* toxin A using a fiber optic-based biosensor. Anal. Biochem. 205, 306–312.

Ogert, R.A., Burans, J., O'Brien, T., Liglers, F.S., 1994. Comparative analysis of tixin detection in biological and environmental samples. Proc. SPIE. Int. Soc. Opt. Eng. 2068, 151–158.

Parsons, R.G., Kowal, R., LeBlond, D., Yue, V.T., Neargarder, L., Bond, L., Garcia, D., Slater, D., Rogers, P., 1993. Multianalyte assay system developed for drugs of abuse. Clin. Chem. 39, 1899–1903.

Plowman, T.E., Durstchi, J.D., Wang, H.K., Christensen, D.A., Herron, J.N., Reichert, W.M., 1999. Multiple-analyte fluoroimmunoassay using an integrated optical waveguide sensor. Anal. Chem. 71, 4344–4352.

Pritchard, D.J., Morgan, H., Cooper, J.M., 1995. Simultaneous determination of follicle stimulating hormone and luteinising hormone using a multianalyte immunosensor. Anal. Chem. 310, 251–256.

Rowe, C.A., Scruggs, S.B., Feldstein, M.J., Golden, J.P., Ligler, F.S., 1999a. An array immunosensor for simultaneous detection of clinical analytes. Anal. Chem. 71, 433–439.

Rowe, C.A., Tender, L.M., Feldstein, M.J., Golden, J.P., Scruggs, S.B., MacCraith, B.D., Cras, J.J., Ligler, F.S., 1999b. Array biosensor for simultaneous identification of bacterial, viral, and protein analytes. Anal. Chem. 71, 3846–3852.

Shriver-Lake, L.C., Anderson, G.P., Golden, J.P., Ligler, F.S., 1992. The effect of tapering the optical fiber on evanescent wave measurements. Anal. Lett. 25, 1183–1199.

Silzel, J.W., Cercek, B., Dodson, C., Tsay, T., Obremski, R.J., 1998. Mass-sensing, multianalyte microarray immunoassay with imaging detection. Clin. Chem. 44, 2036–2043.

Tempelman, L.A., King, K.D., Anderson, G.P., Ligler, F.S., 1996. Quantitating staphylococcal enterotoxin B in diverse media using a portable fiber optic biosensor. Anal. Biochem. 223, 50–57.

Thompson, H.G., Lee, W.E., 1992. Rapid immunofiltration assay of *Francisella tularensis*. Defence Research Establishment Suffield. Can. Suffield Memorandum 1376, 1017.

Wadkins, R.M., Golden, J.P., Ligler, F.S., 1997. Patterned planar array immunosensor for multianalyte detection. J. Biomed. Optics 2, 74–79.

Wadkins, R.M., Golden, J.P., Pritsiolas, L.M., Ligler, F.S., 1998. Detection of multiple toxic agents using a planar array immunosensor. Biosens. Bioelectron. 13, 407–415.

Wijesuria, D.C., Anderson, G.P., Ligler, F.S., 1994. A rapid and sensitive immunoassay for bacterial cells. In: Berg, D.A., Williams, J.D. Jr., Reeves, P.J. (Eds.), Proc. 1993 ERDEC Scientific Conference on Chemical Defense Research, 16–19 November 1993, Report No. ERDEC-SP-024 August 1994, pp. 671–677.