



Technical note

Synergetic chemiluminescence and label-free dual detection for developing a hepatitis protein array

C. Pereira^a, A. Yalçın^{b,1}, M. Cretich^c, M. Chiari^c, M.S. Ünlü^b, D. Nunes^d, D.A. Bergstein^{a,*}^a Zoiray Technologies Inc., Boston, MA, USA^b Department of Electrical and Computer Engineering, Boston University, Boston, MA, USA^c Istituto di Chimica del Riconoscimento Molecolare, C.N.R., Milano, Italy^d Section of Gastroenterology, Boston Medical Center, Boston University, Boston, MA, USA

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ABSTRACT

A dual detection system for protein arrays is presented that combines label-free detection by optical interference with chemiluminescence. A planar protein array that targets hepatitis B surface antigen is developed. Surface densities for individual antibody spots are quantitated using optical interference prior to use. Target binding (10 ng/ml) is detected label-free. Target binding (1 ng/ml) is detected by both optical interference and chemiluminescence with the inclusion of secondary antibodies. Binding results using both methods are found to be directly proportion to the capture probe density measured initially. The dual detection system provides the analytical utility of optical interference detection with the established clinical utility of chemiluminescence detection.

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1. Introduction

The transition to multiplexed immunoassays drives demand for improved analytical tools to more efficiently develop, test, and quality control new array-based diagnostics. Protein microarrays offer the potential to monitor an entire panel of disease markers using a small volume of patient sample in a single test (Cretich et al., 2006). Liver disease is one application that can benefit from multiplexed testing. For hepatitis B alone, important diagnostic and prognostic value is gained from testing for the presence of surface antigen, e antigen, and alanine aminotransferase as well as antibodies against surface antigen, e antigen, and core antigen in patient serum (Gitlin, 1997). With the existing approach, immunoassays are run

separately and add linearly to cost. Furthermore, separate tests, procedures, and reagents to complete a routine panel are factors that inhibit point-of-care testing and favor the common practice of outsourcing panels to centralized labs. The transition to multiplexed methods is paradigm shifting both in that it can enable broad testing for relevant disease markers with little incremental cost and that it can make point-of-care testing more amenable.

With multiplexed arrays come new challenges in assay development and array production. Combining tests requires that the individual immunoassays do not cross-react or otherwise negatively impact one another. In a typical sandwich assay, a primary antibody is immobilized to the solid support, targets selectively bind during a primary incubation, and results are detected via one or more secondary antibodies that specifically introduce a fluorescent or chemiluminescent tag (Cretich et al., 2006). With multiple probes per target, the complexity and potential for cross-reactivity or interference among binders grows rapidly with array size (Ellington et al., 2010). Furthermore, unlike DNA arrays, for which optimized

Abbreviations: IRIS, interferometric reflectance imaging sensor.

* Corresponding author. Tel.: +1 617 358 6003; fax: +1 617 849 5813.

E-mail address: DBergstein@zoiray.com (D.A. Bergstein).¹ Presently at: Department of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, MA, USA.

parameters can often be extended to unique arrays, protein probes and targets vary significantly in their physical properties and tend to work best in individually optimized conditions. Assessing the impact of each step in the immunoassay process and being able to monitor variations are paramount to efficient assay development and quality array production. One important source of variation is the amount of capture protein immobilized to the solid support (Ellington et al., 2010). Different proteins in different buffers under different conditions may covalently link an activated surface more or less efficiently yielding different amounts of probe after washing. Even when trying to maintain an optimized set of production parameters, variations in immobilized probe density can arise easily from small variations in any one of the production variables.

Compounding these difficulties is the fact that for labeled sandwich assays, readout is only achieved during the last step of the process. Using labels generally requires further incubations, binding reactions, and washing steps that can obfuscate analysis of individual steps. Labeled surrogates may be used to characterize surfaces (Nath et al., 2008), though surrogates may not be representative of the actual probes used.

Label-free detection methods such as surface plasmon resonance and optical interference measure binding directly and avoid secondary reactions required for labeling (Ozkumur et al., 2009; Ray et al., 2010). Label-free detection can be highly quantitative and provide real-time binding curves for calculating reaction kinetics and affinity constants (Ozkumur et al., 2010). However, label-free sensitivity (Ray et al., 2010) has generally lagged that of fluorescence or chemiluminescence (Dodeigne et al., 2000), which is of primary concern for many clinical applications. And while label-free detection excels as an analytical tool, commercial label-free systems have required specialized formats and/or surfaces that do not translate easily to improved understanding of a given clinical array process.

We present a dual detection method for planar protein arrays that combines label-free optical interference detection and chemiluminescence detection. The combination presents a way to leverage the strengths of each approach toward improved multiplexed assay development and production. Label-free interference detection by the interferometric reflectance imaging sensor (IRIS) is a simple, highly quantitative, and highly scalable method (Ozkumur et al., 2009; Daaboul et al., 2011). Chemiluminescence detection can achieve remarkable sensitivity and is widely used for clinical hepatitis immunoassays (Chen et al., 2006). The dual detection method is illustrated in Fig. 1. An example that combined label-free and fluorescence detection has been reported in the literature based on the BioCD platform; however, the focus was on improved specificity using both channels (Wang et al., 2008). We have developed a method that combines IRIS and chemiluminescence detection with a focus on its use for assay development and quality control. We apply this approach toward the development of a diagnostic array for liver disease.

2. Materials and methods

2.1. Dual detection instrument

The IRIS method for label-free detection using optical interference has been described previously (Daaboul et al.,

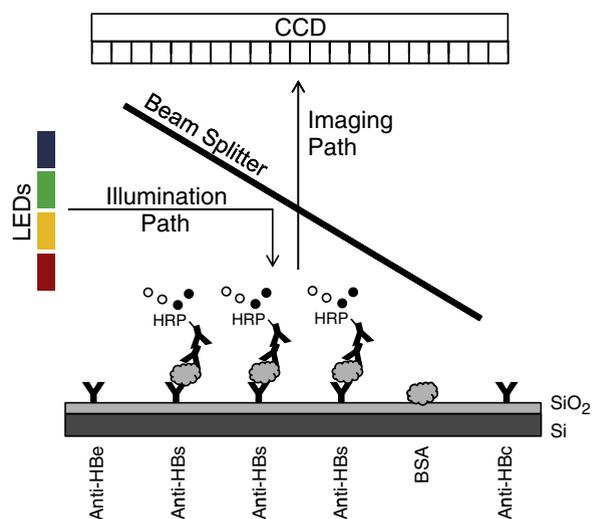


Fig. 1. Dual detection system. Four LEDs illuminate the surface sequentially at 450, 518, 598, and 635 nm. The illumination path includes diffusing optics and a condensing lens (not shown) to homogenize and direct the incident light to the sample via a beam splitter. Either reflected light, in the case of label-free measurements, or chemiluminescence is imaged (lens not shown) on the CCD camera.

2011). The surface in the present case consists of a 500-nm layer of SiO₂ on a Si support. Four light emitting diodes (LEDs) at 450, 518, 598, and 635 nm illuminate the sample in sequence. At each wavelength, light reflects from both the top surface and the buried SiO₂–Si interface. The optical phase difference between the reflections from each surface results in varying levels of constructive or destructive interference at different wavelengths. For each wavelength, the reflection intensity from the entire surface is recorded using a CCD camera. The reflectivity data is fit to a mathematical model and used to calculate the optical path length difference (OPD) between the top surface and the buried SiO₂–Si interface with 5- μ m resolution as previously reported (Ozkumur et al., 2009). Surface binding increases the OPD causing a measurable shift in the reflectivity as a function of wavelength.

Chemiluminescence is recorded by the CCD camera on the same instrument. Some instrument components were selected to accommodate chemiluminescence detection. An enclosure prevents ambient light from entering the system, and the LEDs can be switched off to further provide a dark environment. The CCD camera (QICAM Fast, QImaging) is cooled to reduce noise over longer exposures (typ. 30 s). The array surface is imaged 1:1 to 1392 \times 1040 square pixels, each 4.65 μ m in either dimension, with a numerical aperture of 0.25.

2.2. Surface preparation

Silicon slides with a 500 nm thermal oxide coating (Zofiray Technologies) were treated with a previously described polymeric coating (Pirri et al., 2004). Clean slides were slightly etched in 0.1 M NaOH for 30 min and immersed in copoly(DMA-NAS-MAPS) solution (1% w/v in 0.9 M (NH₄)₂SO₄ water solution) for 30 min. Slides were rinsed with de-ionized water (DI-H₂O), dried with argon, kept at 80 °C for 15 min, and then stored under vacuum until use. The

polymeric coating covalently links capture antibodies to the surface via a non-conformal 3D structure, which maintains antibody activity and resists non-specific binding during target incubation.

2.3. Reagents

Affinity purified hepatitis B surface antigen subtype ad (HBsAg) was used as the target (Fitzgerald Industries International). Solid phase antibodies against hepatitis B core antigen, HBsAg, and hepatitis B e antigen (anti-HBc, anti-HBs, and anti-HBe respectively) were mouse monoclonal (Fitzgerald). Bovine serum albumin (BSA) was immobilized on the arrays as an additional control (Thermo Fisher Scientific). HBsAg was detected with a goat polyclonal antibody targeting human HBsAg (Fitzgerald) and with an anti-goat rabbit polyclonal antibody conjugated to horseradish peroxidase (anti-goat IgG-HRP) (Abcam). SuperSignal Pico West substrate was used for enhanced chemiluminescence (Thermo).

2.4. Arraying

Arrays were printed one per slide in 6×6 grids of 150- μm diameter spots using a robotic spotter (Calligrapher, Bio-Rad). The following capture probes were prepared in phosphate-buffered saline (PBS) and spotted from top to bottom with the printing concentrations indicated parenthetically: anti-HBe (0.3 mg/ml), anti-HBs (1.0 mg/ml), anti-HBs (0.3 mg/ml), anti-HBs (0.1 mg/ml), BSA (0.3 mg/ml), and anti-HBc (0.3 mg/ml). Spotted slides were kept overnight at room temperature in a humid environment. Slides were washed extensively in PBST (0.1% Tween 20 v/v PBS) for 45 min on a lab shaker, replacing solutions every 15 min. Arrays were then rinsed with PBS for 5 min twice, followed by DI-H₂O for 30 s, and dried under argon. An additional slide was printed with anti-goat IgG-HRP in a similar manner for a qualitative signal comparison.

2.5. Incubations and measurements

IRIS measurements were taken dry and consisted of capturing and averaging 100 frames with a 20-ms exposure time at each wavelength. Total IRIS measurement time including data transfer was less than 1 min. Incubations were all performed in 5 ml of PBS solution on a lab shaker for 2 h. Target incubations were with 0, 1, and 10 ng/ml of HBsAg in PBS. Secondary incubations were with 100 ng/ml of goat anti-HBs and 100 ng/ml of anti-goat IgG-HRP. Chemiluminescence measurements were taken after all IRIS measurements using a single 30-s exposure through a small volume (20 μl) of substrate solution.

2.6. Analysis

Spots were analyzed for both measurement channels by averaging the signal from pixels inside individual spots (typically 1000 pixels) and subtracting from it a background signal, which was defined for each spot by the average signal in an annulus (typically 1000 pixels) around the spot. Within averaged areas, the lowest and highest 2.5% of pixel values (typically 50 pixels) were removed from pixel averages as

outliers. Analysis was carried out using the ZoirayProcess software package (Zoiray). Values reported for each row of spots are the mean of the signals from the 6 repeated spots and their standard deviation.

3. Results and discussion

3.1. Instrumentation

The dual detection instrument contains relatively few components and no moving parts. The beam splitter, used for IRIS measurements, remained in place and reduced the chemiluminescence signal about 50%. A moveable beam splitter could be used to avoid this signal loss. The signal is increased 2-fold by the by the SiO₂-Si slides as compared to glass (data not shown). A two-fold improvement is expected due to the reflective nature of the SiO₂ on Si structure (the effects of interference are not pronounced given the short coherence length of the chemiluminescence in relation to the 500-nm layer thickness).

3.2. Test design

Hepatitis B surface antigen (HBsAg), which appears in sera during the early stages of infection (Gitlin, 1997), served as the target for the present demonstration. Control spots, used to demonstrate specificity, included anti-HBc, anti-HBe, and BSA. To simplify matters further, only one target-specific antibody was used for secondary incubation: polyclonal goat anti-HBs. For a full hepatitis assay, multiple specific secondary probes would be required, one for each target. To avoid many separate HRP conjugations to secondary probes, a final antibody with broad specificity is used to link HRP to the binders. Incubations were performed in PBS to avoid possible cross-reactivity and other potential complicating factors patient sera could introduce to the demonstration.

3.3. Probe immobilization

Label-free detection provides an ability to evaluate the mass bound during each step of the assay. Fig. 2 provides a highly visual demonstration of the correlation between the dual signal channels using spotted IgG-HRP as a surrogate probe. Though the enhanced chemiluminescence substrate is in solution, image resolution was fine enough to observe spot morphology. The IRIS resolution is greater and spot features in the profile appear sharper. The IRIS signal (left vertical axis in Fig. 2) has been previously shown to provide a quantitative measure of protein mass with a 6 pg/mm² noise floor (Ozkumur et al., 2009). The noise floor was confirmed for the present system and corresponds to 0.2 pg over one spot. The chemiluminescence gives a relative level for the amount of HRP at each location (right vertical axis in Fig. 2). The pronounced variation and morphology of the three spots shown helps illustrate the qualitative correlation between the two signal channels.

For the present assay, the ability to detect the surface probe mass was useful for developing the washing procedure. A less aggressive washing procedure consisted of washing slides twice in PBST for 5 min and twice in PBS for 5 min. At a spotting concentration of 1 mg/ml, an average of 177 pg of

anti-HBs antibody was detected at spots post-washing. After repeated washings, the IRIS signal was found to decrease variably across spots by 10–40%. Removal of probe molecules loosely adsorbed on the surface when the surface is incubated with the target may represent a serious problem in terms of reproducibility of the signal detected. A more aggressive washing procedure that stabilizes the amount of probe immobilized is preferable. IRIS detection provided a means to assess the point at which the immobilized mass no longer decreased under operative conditions. After an extensive wash, 43 pg of anti-HBs was measured for spots that had been printed with a spotting concentration of 1 mg/ml. This value changed less than 3.5 pg under control conditions with buffers that are typically used for target incubation. Assuming a pure spotting concentration, 43 pg is equivalent to 1.7×10^8 molecules of the 150-kD antibody.

3.4. Target binding

IRIS measurements were again performed after the target incubation. HBsAg binding was undetected for both the control slide and for 1 ng/ml HBsAg (signals remained close to 0 with a standard deviation less than 2 pg). For 10 ng/ml HBsAg, binding was detected label-free for the highest density anti-HBs row with an average IRIS signal (\pm one standard deviation) of 4.9 pg (± 0.8 pg). Given that the target solution was highly pure,

4.9 pg equates to 1.2×10^8 molecules for the 25-kD protein. Comparing this value with the calculated number of antibodies in the previous section indicates there is nearly one antigen per antibody. This level of antigen binding might at first appear high since not all the surface antibodies can be expected to be active; however, the expected affinity is strong (10^{10} M^{-1}), antibodies are by their nature bivalent, and surface antigen is known to aggregate (Koistinen, 1980).

3.5. Secondary antibody binding

All slides were incubated with both secondary antibodies and detected using IRIS and enhanced chemiluminescence. For the control slide, measurements remained close to zero with an average increase of 0.2 pg (± 1.5 pg) for IRIS measurements and 15 RLU (± 4 RLU) for chemiluminescence (RLU=relative light unit). While 1 ng/ml was undetected after the target incubation, it was clearly visible after incubation with the polyclonal anti-HBs (150 kD) and the anti-goat IgG-HRP (200 kD) which boost the IRIS signal in what can be considered mass labeling. Final IRIS and chemiluminescence results for 1 ng/ml HBsAg are reported in Fig. 3. In the figure, the scaled left and right axes, reporting the IRIS signal and chemiluminescence, respectively, show the strong correlation between the final results using the two signal channels.

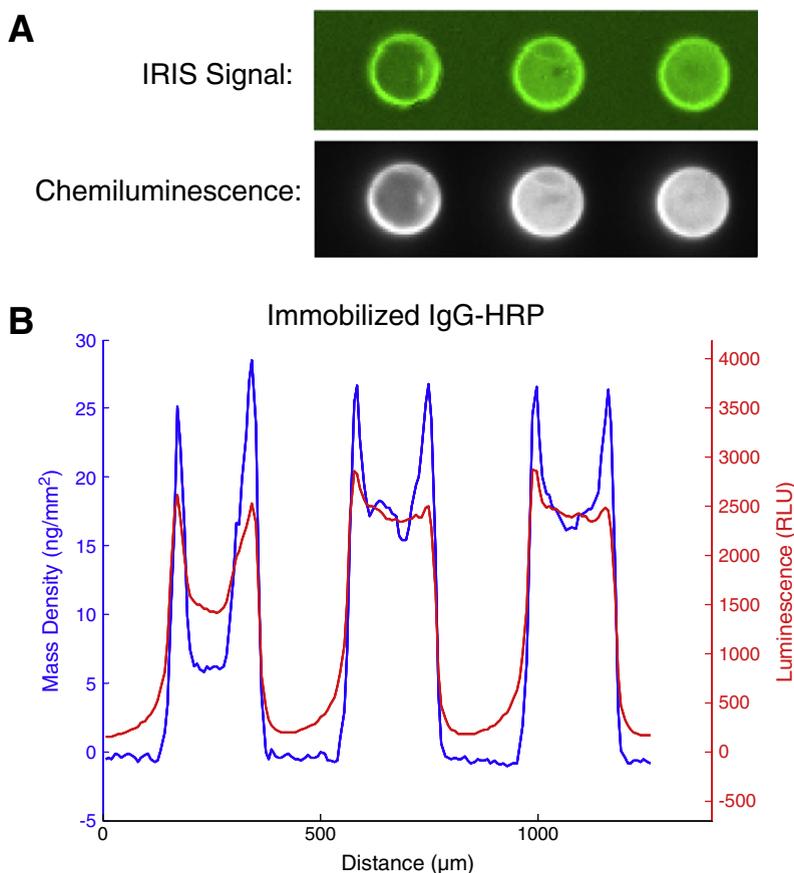


Fig. 2. Qualitative comparison of IRIS and chemiluminescence signals. (A) Images of the IRIS and chemiluminescence signals from three spots. (B) Line profiles of the three spots. IRIS signal is expressed as mass density (left vertical axis) and the chemiluminescence is expressed as relative light units (right vertical axis).

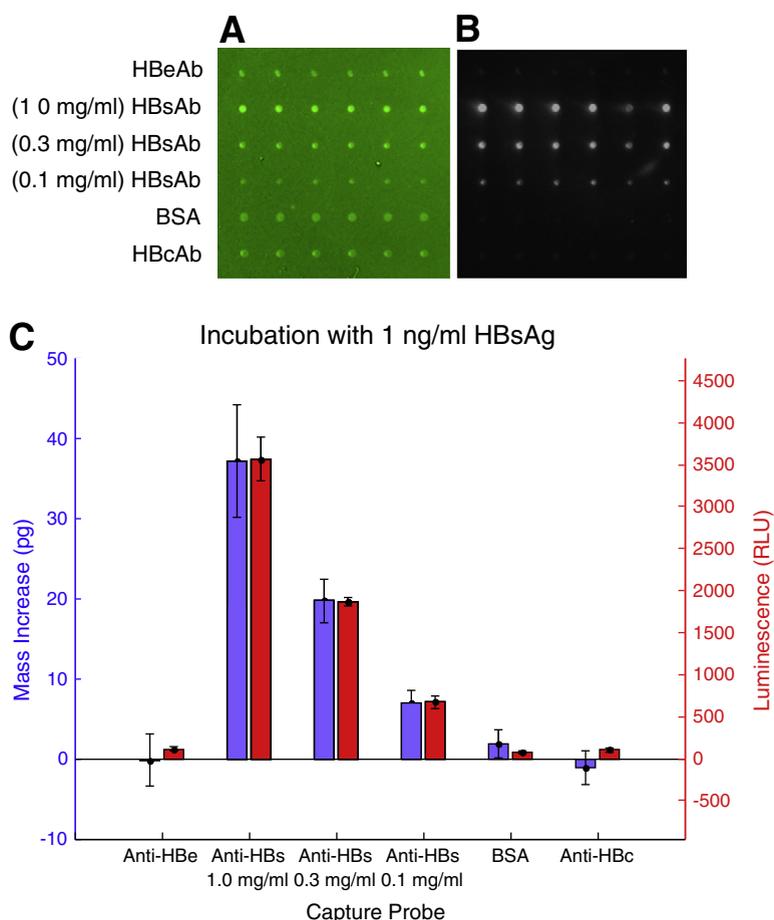


Fig. 3. Results for 1 ng/ml HBsAg detection. (A) Final IRIS image. All spots contain at a minimum their capture probe and are hence all visible in the image. (B) Image of chemiluminescence result. (C) Binding signals for each row. The mass increase after incubation with the target and secondary antibodies was measured by IRIS and indicated on the left vertical axis. Resulting chemiluminescence intensity is given by the right vertical axis as relative light units.

The results, both IRIS signal and chemiluminescence, were further evaluated in relation to the initial IRIS measurement. The final signal in each anti-HBs row was found to be directly proportional to the average immobilized antibody mass for that row. The average immobilized mass was 43.4, 19.4, and 9.8 pg for the three anti-HBs rows spotted with 1, 0.3 and 0.1 mg/ml, respectively. Looking at the final IRIS signal, the ratio of increased mass to immobilized mass was 0.86 ± 0.16 . Looking at the chemiluminescence, the ratio of intensity to immobilized mass was $82 \text{ RLU/pg} \pm 14 \text{ RLU/pg}$. These values suggest a linear relationship between the immobilized probe mass and spot binding capacity over the range of probe densities tested. From a quality control perspective, initial IRIS measurements could be used to accept or reject individual spots or entire arrays based on probe density to reduce this source of variability.

3.6. Dual detection flexibility

The dual detection system presented enables high density arrays to be developed using label-free detection as an analytical tool on a platform that is, at the same time, amenable to becoming a clinical diagnostic platform using labeled detection

without necessitating a change in surface, chemistry, or format. The dual detection instrument is relatively simple and inexpensive in its construction, as is the production of the disposable slides. Slides feature a SiO_2 surface that is compatible with a wide variety of surface chemistries including the polymer selected here. Clinical immunoassay systems for testing HBsAg today achieve a limit-of-detection of 0.2 ng/ml in sera using enhanced chemiluminescence (Chen et al., 2006). Though a formal limit-of-detection was not determined, we believe the enhanced chemiluminescence signals shown for 1 ng/ml of HBsAg are evidence that clinically relevant sensitivities can be achieved. The IRIS signals shown for 1 ng/ml with mass labeling were observed to have larger error bars, suggesting chemiluminescence remains the more sensitive detection modality. For some targets, and hence at some spots, label-free sensitivity and specificity without secondary antibodies may be sufficient. Label-free detection in complex sample matrices, however, is complicated by the need to discriminate target binding from non-specific adsorption without the benefit of a secondary antibody that must also bind in order for the target to be identified. That said, secondary antibodies can cause problems of their own if they non-specifically bind primary antibodies in the assay, a challenge that can grow with the level of multiplexing

and the number of different primary and secondary antibodies included. The greatest value of the presented dual detection system, we believe, is the flexibility to apply different detection modalities where most useful for assay development, quality control, and clinical use.

Competing Financial Interests

C. Pereira and D.A. Bergstein are employees of Zoiray Technologies Inc. M.S. Unlu and D.A. Bergstein are directors of Zoiray Technologies Inc. and hold equity positions.

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