axela BIOSENSORS

Solving the multiplexing–dynamic range conundrum with Diffractive Optics Technology (dot™)

The ability of the dotLab™ System to concurrently perform monitoring of protein-protein interactions in real-time, while using different assay formats, overcomes the traditional limitations of multiplexed end-point assays.

Immunoassays have an established role in the assessment of physiological processes in both healthy and disease states. In an effort to obtain a large amount of information from precious complex biological samples, biomedical researchers have attempted to multiplex immunoassays in small sample volumes. It is also becoming apparent that panels of analytes offer greater promise for enabling diagnostics with greater sensitivity, specificity and clinical utility.

Although there have been many approaches to multiplexing immunoassays, the number of biomedical and diagnostic panels has lagged with respect to expectations. It is generally acknowledged that multiplexed assays are inherently difficult to develop owing to potential cross-reactivities between reagents aimed at different analytes. Present solutions for this problem have relied on time-consuming, laborious and expensive approaches to optimization of assays and reagents. Additionally, multiplexed assays typically have limited dynamic range. In fact, most assays require dilution of the sample to accommodate high-concentration analytes, which can in turn lead to loss of signal for low-level analytes. A closer look reveals that most panels are grouped according to concentration range, not necessarily by their utility.

The dotLab System overcomes these shortcomings by providing a more robust and accessible approach to reagent optimization as well as providing a flexible platform for multiplex immunoassay with a broad dynamic range.

Principles of diffraction

Analysis is carried out on the surface of a dotLab Sensor. Each sensor is manufactured with eight assay spots along a linear flow channel (**Fig. 1**). The dotLab System introduces samples and assay reagents into the dotLab Sensor using an automated sampling system and a high-precision fluidic controller. Binding of target molecules to the patterned assay spots is detected by interrogation with focused laser light in a total internal reflection mode. Binding events cause an increase in the diffraction signal, which is detected in real time using photodiodes. The detection beam never passes through the flow channel, providing an ideal platform to work with complex biological samples.

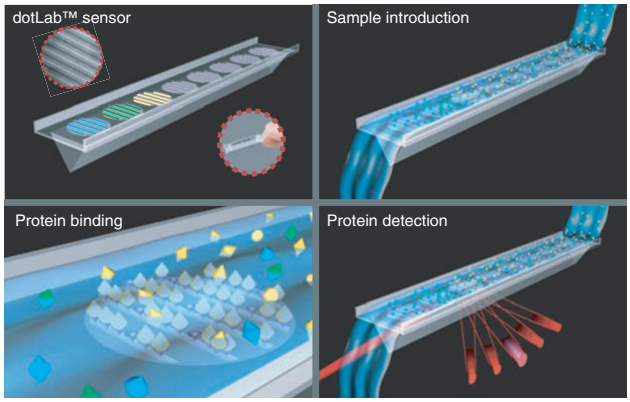

Figure 1 | dotLab basics. Capture molecules are deposited onto the sensor surface as a diffraction grating. Eight individual spots can be patterned in this fashion. Test solutions are introduced into the sensor, and as analytes bind on the surface, the efficiency of the diffraction grating improves. As the grating is interrogated during the binding reaction, an increase in diffraction signal is measured in real time.

It is important to note that diffraction is inherently self-referencing: because the transduction of binding events is dependent on the initial pattern, an increase in diffractive signal intensity will only occur if molecules bind exclusively to the patterned capture reagents. In other words, nonspecific binding to both the patterned and nonpatterned regions will not affect the signal substantially. This attribute provides a considerable advantage over other optical biosensor systems in which any surface binding event will cause an increase in signal. Finally, there is virtually no background signal from adjacent assay spots, unlike with fluorescence or other label-based approaches. In these approaches, signal emanating from nearby features or beads can contribute to background and overwhelm adjacent, low-concentration measurements.

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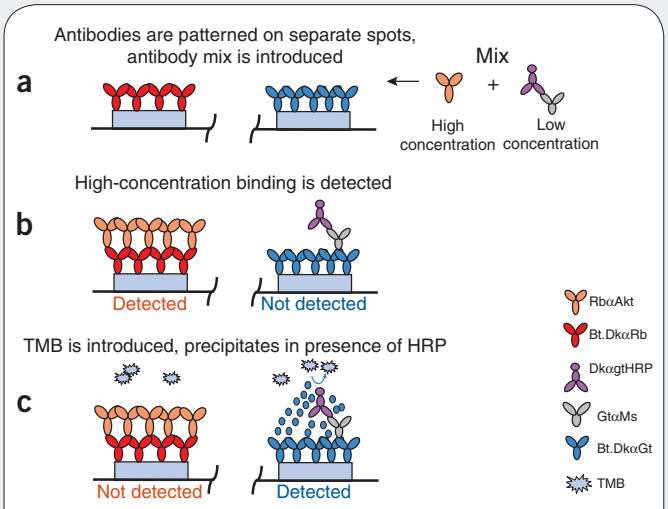

Figure 2 | Schematic representation of each complex formed. (**a**) Bt.DkαRb and Bt.DkαGt were patterned on separate spots on a single sensor. RbαAkt is present at 10 μg/ml and GtαMs is present at 10 pg/ml in the antibody mix that is loaded onto the sensor. (**b**) RbαAkt is detected, binding to the surface, whereas GtαMs/DkαGt-HRP, in minute concentrations, provides no detectable signal. (**c**) TMB is introduced into the sensor and is precipitated by HRP conjugated to the DkαGt; thus, the complex is detected.

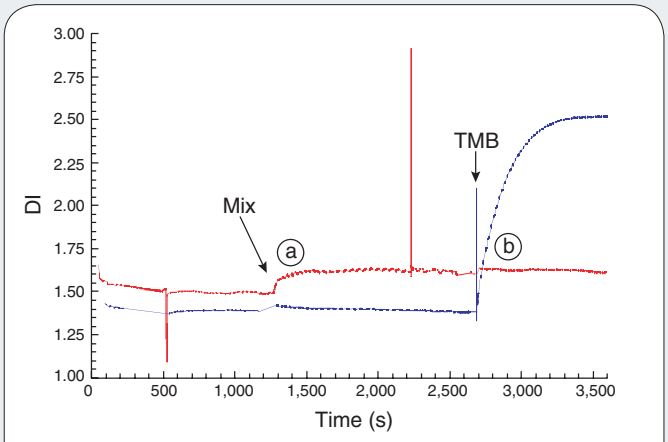

Figure 3 | Concurrent detection of analytes over six orders of dynamic range. (**a**,**b**) The specific detection of each complex is shown in the schematic in **Figure 2**. After introduction of the antibody mix, the binding of the RbαAkt antibody to Bt.DkαRb (**a**) is detected by the rise in signal on the red trace. Note the lack of signal on the blue trace, indicating no cross-talk with Bt.DkαGt. The binding of the very-low-concentration analyte is not immediately detectable. For the GtαMs/DkαGt-HRP complex, an additional step involving the specific and localized precipitation of TMB, mediated by HRP, is required (**b**). The spikes in the traces represent air gaps that are used to separate reagents and also help mark the introduction of reagent or sample. Analyte signal is presented in diffraction intensity (DI) units.

We have previously demonstrated the versatility of the system in optimizing reagents and assay parameters, as well as the implementation of a variety of immunoassay formats to permit direct measurement over a broad dynamic range[1]. These combined assay approaches are incompatible with traditional endpoint and label-based methodologies. In the cited example we used this multiple format approach to detect NT-proBNP, a cardiac biomarker in micromolar, nanomolar and picomolar concentration ranges. Here we demonstrate the simultaneous quantitation of two similar analytes present at concentrations that differ by over six orders of magnitude.

Multiplex analysis

We used a mixture of a rabbit anti-akt (Rbαakt), goat anti-mouse (Gtαms) and horseradish peroxidase–conjugated donkey anti-goat (Dkαgt-HRP) to demonstrate analyte detection over a wide dynamic range. Rbαakt served as the high-concentration analyte at 10 μg/ml. Gtαms served as the lower-concentration analyte at 10 pg/ml. We immobilized two biotinylated donkey antibodies on the surface of two different assay spots of an eight-spot streptavidin dotLab Sensor (**Fig. 1**). We immobilized a biotinylated donkey anti-goat (Bt.DkαGt) antibody on one spot and a biotinylated donkey anti-rabbit (Bt.DkαRb) antibody on a separate assay spot. The binding events occurring at each of these locations were recorded. The anticipated interactions are indicated in the schematics in **Figure 2**.

The traces in **Figure 3** are a representative example of this multiplexing experiment. The binding of the very-low-concentration analyte is not immediately detectable: for low-level analytes, an additional step involving the addition of 3,3′,5,5′-tetramethylbenzidine (TMB) is required. The specific and localized precipitation of TMB, mediated by HRP, on the diffraction pattern causes a signal increase (**Fig. 3**). Furthermore, the signal increase is detected solely on the anticipated assay spot (blue trace). Therefore, specific detection of the two analytes was accomplished over six orders of concentration without signal cross-talk.

Conclusion

The use of dotLab Sensors and the dotLab System for multiplexing the detection of analytes present over a wide dynamic range has been demonstrated. This is made possible by the unique coupling of flow-based sensors, independent multiplex analysis and real-time measurements generated throughout the entire assay process. This combination allows a wide breadth of assay formats to be run on the same sample and results in the unprecedented dynamic range. Independent analyte detection obviates the need to compromise on most assay parameters demanded by multiplexed endpoint technologies. Furthermore, analyte determinations can be based on binding-rate measurements, shortening the time to result. The dotLab System uniquely permits the development and use of new multiplexed panels, resulting in greater analytical and clinical utility. This capability is now being applied to a range of biomarker combinations, in which limited sample volume or wide concentration ranges previously prevented simultaneous measurement.

Additional information may be found at http://www.axelabiosensors.com.

ACKNOWLEDGMENTS

We recognize D. Wong of Axela Biosensors, Inc. for his assistance in developing this application note.

1. Borisenko, V. *et al.* Diffractive optics technology: a novel detection technology for immunoassays. *Clin. Chem.* **52**, 2168–2170 (2006).