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“Spot and Hop” – Internal Referencing for Surface Plasmon Resonance Imaging Using a Three-Dimensional Microfluidic Flow Cell Array

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Abstract

We have developed a novel referencing technique for surface plasmon resonance imaging systems, referred to as “spot and hop”. The technique enables internal referencing for individual flowcells in a parallel-processing microfluidic network. Internal referencing provides the ability to correct for nonspecific binding and instrument drift, which significantly improves data quality at each region of interest. The performance of a 48-flowcell device was demonstrated through a series of studies, including rise-and-fall time, ligand preconcentration, ligand immobilization, analyte binding, and regeneration tests. Interfacing parallel-processing fluidics with imaging systems will significantly expand the throughput and applications of array-based optical biosensors while retaining high data quality.

Keywords

Protein array; Biacore; Flexchip; SPR; kinetics

Introduction

While standard surface plasmon resonance (SPR) biosensors have become widely adapted for molecular interaction analysis, Imaging systems such as those available from Biacore (Flexchip), Genoptics, GWC, IBIS, and Maven have had less of an impact to date. Imaging systems typically involve patterning spots of ligands within a large flow cell and then testing the binding of a single analyte at a time, the so-called ‘one-on-many approach’ [1].

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Two issues limit the widespread applications of imaging systems. The first challenge is how to pattern protein ligands onto the sensor surface. The majority of imaging instruments rely on pin-spotting, ink-jet printing, and stamping methods, which do not work well for many real-world applications [2–5]. The necessity to immobilize outside of the flowcell often leads to lengthy optimization studies and/or irregular spots and requires high concentrations of ligands.

To address this first problem we have developed a three-dimensional microfluidic flowcell array (MFCA) [6–8] (see Figure 1A). This is a three-dimensional device that can deposit 48 isolated spots in a 4 mm × 10 mm area [7]. A close-up image of the flow cell shape and pattern is shown in Figure 1B. The advantage of a 3-D configuration over existing 2-D configurations is the ability to increase the density of spotting regions by having isolated spots, not flow lanes. The MFCA dramatically outperformed pin-spotting in comparison tests making it possible to deposit ligands that are dilute as well as in crude solutions [7,8].

The second issue limiting the widespread adoption of imaging systems is the limited number of applications for the one-on-many assay approach. Most users in fact want to run the reverse assay: they have many analytes to test against one, or a few, ligand(s). Drug discovery is a good example. In these cases, the problem is not with the imaging technology but rather with sample delivery.

In this report, we demonstrate how our MFCA devices can be used to create independent yet parallel processing flow cells when interfaced with imaging systems. We also introduce the concept of “spot and hop” as a simple yet novel method of creating both a reaction and reference surface within each flow cell. The current device incorporates 48 isolated flow cells that can be used to deliver different analytes to the sensor surface. Using Protein A/IgG as a model system, we demonstrate the effectiveness of the MFCA system as well as the benefits of internal referencing. Interfacing parallel-processing microfluidics with imaging technology will expand the use of array biosensor technology in drug discovery, diagnostics, and biomarker detection.

Materials and Methods

Standard MFCA printheads and an instrument were manufactured by Wasatch Microfluidics (North Salt Lake, UT). The imaging detector, a Proteomic Processor, was acquired from Plexera (Seattle, WA). Protein A and bovine IgG were acquired from Sigma-Aldrich (St. Louis, MO). CM5 chips were purchased from GE Healthcare (Uppsala, Sweden). The response data were processed using Scrubber2 (Biologic Software Pty Ltd, Australia).

MFCA-Imaging Integration

Custom fixtures were adapted to the Proteomic Processor top plate to mount the MFCA above the sensor platform. Positioning x-y-z stages (Edmunds Optics, Barrington, NJ) were used to provide control of the MFCA for the spot and hop movement. A MFCA instrument was adapted to provide the fluidic control for all 48 flow channels in the MFCA. A CM5 sensor chip was docked between the MFCA and Proteomic Processor's optical detector.

Rise and fall time test

Running buffer (PBS, pH 7.4) was initially pumped through all 48 flow cells for 5 minutes at a flow rate of 150 μ L/min to establish the baseline. A 5% solution of glycerol was then injected at the same flow rate for 10 minutes, followed by buffer for another ten minutes.

Protein A preconcentration test

The flow system was equilibrated with PBS for 10 minutes prior to injecting a 10 μ g/ml solution of Protein A in 10 mM sodium acetate at pH 5.0. The flow rate was held constant at 150 μ L/min. The Protein A solution was injected for six minutes, followed by PBS buffer.

In situ activation, immobilization, binding and regeneration

The *in situ* amine coupling was accomplished using a 1 to 10 dilution of a mixture of N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS). The mixture was injected at 150 μ L/min for 6 minutes. Protein A at 10 μ g/ml in 10 mM sodium acetate pH 5.0 was then injected for six minutes, followed by inactivation of the ester active sites by ethanolamine (1 M, pH 8.1) for an additional six minutes. Buffer was then injected for six minutes to stabilize the baseline prior to antibody binding. Bovine IgG at 300 nM was used as an analyte. Regeneration steps were performed using HCl at dilutions of 1 to 1000 or 1 to 500.

Spot and hop test

Protein A was immobilized using amine coupling as described above onto a CM5 sensor chip. One channel was left blank without immobilization as a control. After immobilization, all liquid was removed from the MFCA. The MFCA tip was retracted and moved horizontally 0.3 mm, then redocked onto the sensor surface such that each flow cell now contained a reaction and reference region of interest. Signals from the reaction and reference spot regions were set at 50×50 m squares. PBS buffer was injected to reestablish a baseline. Bovine IgG (300 nM) in 0.5% glycerol was then injected for seven minutes at 150 μ L/min to intentionally introduce a bulk refractive index change during the binding study. The bound IgG was removed with a subsequent regeneration step (1 to 500 dilution of HCl).

Results

We integrated our 48 flow cell MFCA device with the SPR detector in a Proteomic Processor. Figure 2 shows an SPR image of the chip surface. The dark regions represent the flow cell chambers of the 48 flow channels, which appear in sharp contrast to the white background which is where the MFCA docks with the sensor chip. The image itself is compressed slightly in the horizontal dimension due to the optics of the imaging system. Note how the shapes and positioning of the flow cell image match the physical structure of the fluidic device shown in Figure 1.

To test the performance of the 48 flow cell system, we started with a basic rise-and-fall time experiment. As shown in Figure 3, we flowed buffer through the system for five minutes before switching to a sample of running buffer containing 5% glycerol in 47 of the channels. At a flow rate of 150 μ L/minute we observed a sharp transition in response at time 0, which

indicated the start of the injection. The responses from the 47 channels rapidly reached equilibrium and maintained a steady response until 600 seconds, at which point the sample was switched back to running buffer and the signal decayed back to baseline. The response in the single channel without injecting the glycerol sample remained flat throughout the experiment (see bottom response in Figure 3).

Having demonstrated through the rise-and-fall time test that the fluidics were performing well across the entire flow cell array, next we wanted to demonstrate the ability to carry out chemical immobilization steps with this system. To do this, the first thing we needed to show was that the dextran-coated CM5 sensor chips, which we used as the substrate slide, would perform as anticipated for this application.

One of the advantages of using carboxymethyl dextran surfaces is an effect called preconcentration. This occurs when the pH of the ligand solution is low enough that the ligand takes on a positive charge and is electrostatically attracted to the negatively charged surface [9]. For this study, a sample of Protein A was injected through the MFCA at a concentration of 10 ug/ml in 10 mM sodium acetate pH 5.0. As shown in Figure 4, we observed an increase in response as the Protein A was injected over the CM5 sensor chip. The results are nearly identical to a preconcentration study in the first reported use of dextran for SPR [9]. The sigmoidal shapes of the Protein A preconcentration binding responses are consistent with concentration gradients created in the dextran layer as a result of the high capacity these surfaces have for charged proteins. The slight stagger in the responses between flow cells is due to different path lengths within the microfluidic channels. At 230 seconds the flow was intentionally stopped for 10 seconds to observe the change in binding rate under conditions of no flow. After injecting Protein A for six minutes, PBS running buffer was injected through the flow channels and the response rapidly returned to baseline as the Protein A that was preconcentrated on the surface was released by the presence of higher salt (150 mM NaCl) in the buffer. This illustrates that the Protein A was not irreversibly absorbed onto the sensor surface.

Having demonstrated that the fluidics and dextran surfaces were working properly together, we next ran a full *in situ* activation, immobilization, binding, and regeneration study. As shown in Figure 5, we activated the surfaces within 46 of the channels using EDC/NHS for six minutes (area a), followed by an injection of Protein A (in 10 mM NaAcetate) for six minutes (area b) and then a blocking step with ethanolamine for six minutes (area c). This resulted in an average coupling response for Protein A of ~75 response units.

Next we injected bovine IgG at a concentration of 300 nM over the Protein A surfaces (see Figure 5 area d) and detected a significant response that averaged around 300 response units above background. After a 1000 second dissociation phase, the surfaces were washed with 1 to 1000 (area e) and 1 to 500 (area f) dilutions of HCl as a regeneration condition. The binding of bovine IgG was successfully repeated (area g), followed by a subsequent regeneration cycle (1 to 500 HCl, area h), which returned the responses back to baseline. The ability to repeatedly bind IgG to the Protein A surfaces demonstrates that the Protein A is covalently bound to the sensor surface.

Having successfully demonstrated the ability to immobilize ligands in parallel using our microfluidic system, next we illustrate how we can create reference regions within each flow cell. After immobilizing the ligand, the MFCA printhead was withdrawn from the surface and moved horizontally approximately half a spot such that each flow cell would now occupy space over a region with and without ligand. An example of the chip image for four of the spots is shown in the inset of Figure 6A. In these images the lighter regions represent the areas where Protein A was immobilized and the darker regions represent the reference regions.

Once redocked, the chip surfaces were washed for ten minutes with PBS running buffer followed by an injection of bovine IgG (300 nM), which contained 0.5% glycerol to intentionally add a bulk refractive index change to the responses. Figure 6A shows the raw data collected from the reaction and reference regions in each flow cell. Note the significant bulk refractive index change that is recorded from the reference regions and is present in the reaction regions as well. Two flow cells were used as controls. In one, we injected buffer only, which gave no response (see flat lines in Figure 6A) and the second had the IgG sample but no Protein A immobilized and only showed the bulk refractive index change.

Finally, we used the responses from the reference regions to subtract out the bulk refractive index changes and any nonspecific binding or drift that was present in each flow cell. Figure 6B shows the reference-corrected data for the IgG injection. The standard deviation in the binding response was <10% and likely stems for slightly different levels of immobilized Protein A. The two responses with no significant change in signal are from the two reference flow cells.

Discussion

In this report we interfaced our parallel array of 48-microchannel flowcells with a surface plasmon resonance imaging system called the Proteomic Processor. In this case the open format of the Proteomic Processor made it easy to integrate to the prism and chip holder. We used custom fixtures and positioning stages to enable accurate retraction and horizontal movement of the flow cells for the spot and hop experiments, as well as for removing and inserting sensor chips.

The rapid changes in response observed in the rise and fall time experiments demonstrated our system has the potential to be used in kinetic binding studies. We also demonstrated the ability to carryout multi-step immobilization methods using sequential injections of activation, coupling, and blocking conditions. And, we showed that we could reproducibly bind an analyte and regenerate bound complexes. We employed amine coupling on dextran-coated surfaces from Biacore since these surfaces are readily available and this chemistry is commonly used. But, our fluidic system should be able to handle other immobilization methods and surface chemistries.

By repositioning the flow cells after the immobilization step, our spot and hop method makes it possible to create independent reaction and reference regions of interest within each flow cell. We applied the internal referencing step to data collected for the binding of IgG to

immobilized Protein A. The resulting binding responses appeared very consistent between the different flow cells. But, while the responses have a classic shape, the kinetics are in fact complicated by the fact that the interaction of Protein A with IgG is not simply 1 to 1 [10]. However, these data do demonstrate internal referencing can significantly improve the quality of response data by providing the means to remove bulk refractive index changes, instrument drift, and nonspecific binding.

The MFCA-Imaging integrated platform illustrates the potential for a parallel high-throughput detection platform. The ability to test 48 samples at one time opens up a number of unique experimental options. For example, in a screening mode users may test the binding properties of 48 different analytes (e.g., antibodies or even small molecules). The technology could also expand biophysical studies. Because the flow paths are completely independent of one another and because each contains its own internal reference, it is possible to test the binding of one analyte under different buffer conditions (e.g., pH, salt, or detergents).

Given the potential throughput of our system, we are currently working on developing an automated sample introduction station to handle reagents in a 96- and 384-well plate format. We are also looking into the potential of scaling up the number of channels in our device from 48 to 96 independent flow cells to enhance throughput even further. Finally, by incorporating finer motion controls on the docking station we are investigating the potential to spot multiple times within each flow cell.

Conclusion

The MFCA was successfully integrated with an existing commercial imaging platform, the Proteomic Processor making it possible to analyze 48 analyte samples in parallel. We demonstrated good system performance and the ability to perform *in situ* immobilization. Our unique spot and hop approach to referencing can provided a useful tool for reducing system artifacts and improving data quality. Our technology offers the means to capitalize on currently available imaging systems by significantly expanding throughput as well as applications.

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Figure 1.
A. Picture of the tip of a MFCA. B. An SEM image captured at the tip of the MFCA.

Figure 2.
An image captured for a 48 channel MFCA directly coupled to an SPRi instrument.

Figure 3. Rise-and-fall time test. 5% glycerol was injected through 47 channels of a 48 channel system at a flow rate of 150 $\mu\text{l}/\text{min}$.

Figure 4. Preconcentration test of the Dextran surface with Protein A. The protein A response returned to baseline following the buffer injection.

Figure 5.

In situ activation, immobilization, and regeneration in the MFCA-SPRi, a) EDC/NHS activation of Dextran b) Protein A immobilization (10 $\mu\text{g/ml}$), c) ethanolamine inactivation of Dextran, d) captured Bovine IgG (300 nM), e) HCl regeneration (1 to 1000 dilution), f) HCL regeneration (1 to 500 dilution), g) captured Bovine IgG (300 nM), and h) HCl regeneration (1 to 500 dilution).

Figure 6. Spot and hop referencing by creating a spot half activated by protein A and leaving half as inactivated dextran. A) Raw sensorgram from both the reaction and reference regions. The inset show an image of four half activated/half inactivated regions. B) Reference-corrected data.