IBIS White paper # 14.151021

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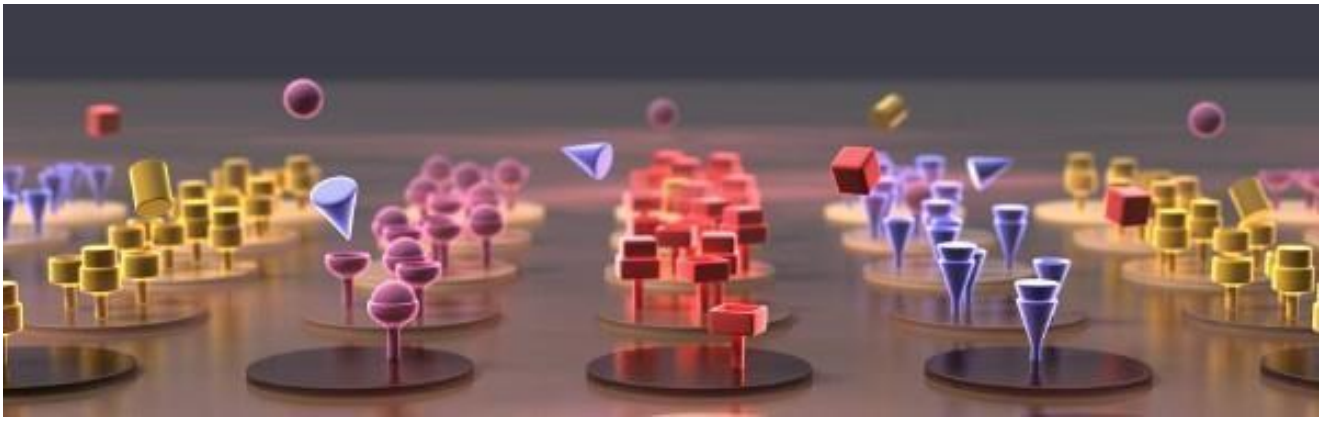

IBIS White paper
First Pass Kinetics™ Screening of Captured Antibodies
 # 14.151021

- ***An array of 96 captured antibody positions was constructed and screened for binding kinetics against IL-6R in a single day using the CFM/MX96 SPR imaging platform.***
- ***Extended capture times enabled kinetic fitting for ligand concentrations varying by more than two orders of magnitude and as low as 0.05µg/ml***
- ***Kinetics values were reproducible across the sensor surface and calculated affinity was within approximately 5-fold of a traditional SPR platform***
- ***Rapid processing and interpretation of arrayed data was achieved using custom fitting and visualization software tools for array-based data sets***

Introduction

Essential to selection of candidate antibodies in drug discovery is the determination of binding kinetics against antigen and calculation of resulting affinity (K_D), frequently accomplished using surface plasmon resonance (SPR) biosensors. Traditionally, SPR platforms have required compromises in throughput and/or level of kinetic detail in order to evaluate the high numbers of antibodies generated through modern expression platforms [1]. To augment throughput, a partial kinetic screen of antigen- antibody at a single concentration is commonly run to avoid both the measure of antibody concentrations for large sample sets as well as the added time and sample requirements for a full concentration series of analyte [2]. Variable expression levels of candidate antibodies can complicate apparent kinetics and/or restrict samples to only those with sufficient concentration for a given assay. Additionally, the use of dissociation rate (k_d) alone ignores the association rate (k_a) and still requires subsequent full kinetics re-assay of selected candidates to determine affinity. A more ideal scenario would be to measure both k_a and k_d rapidly at an early stage in the screening process to facilitate effective ranking of candidates based on specific drug discovery. Using an advanced printhead system, the Wasatch Microfluidics Continuous Flow Microspotter (CFM) couples 96 protein spots discretely on a sensor chip surface which can then be monitored simultaneously for binding in real-time using the IBIS MX96 SPR imager (SPRi). Individually coupling proteins in the CFM gives the option of using up to 96 unique antibodies as ligand or instead using a smaller subset under varying conditions such as concentration or contact time. A unique feature of both the CFM and MX96 is the ability to flow sample across the sensor surface using bidirectional flow, thereby enabling extended contact times for immobilization, capture, and/or binding.

The following is an example of a binding kinetics screening strategy using capture of IL-6R antibody on immobilized anti-IgG Fc surface followed by probing with a serial titration of IL-6R. Varied antibody densities were generated across the chip surface to demonstrate both measures of reproducibility as well as to highlight the dynamic range of this system for looking at kinetics of ligands with broad differences in concentration. This approach to rapid profiling and ranking, termed First-Pass Kinetics™, presents a robust means of screening high numbers of candidates while still enabling the benefits of detailed antigen-antibody binding kinetics.

Materials and Methods

Amine coupling of anti-IgG Fc mAb lawn

Figure 1 gives an overview of the process for creating the captured antibody surface and probing with analyte.

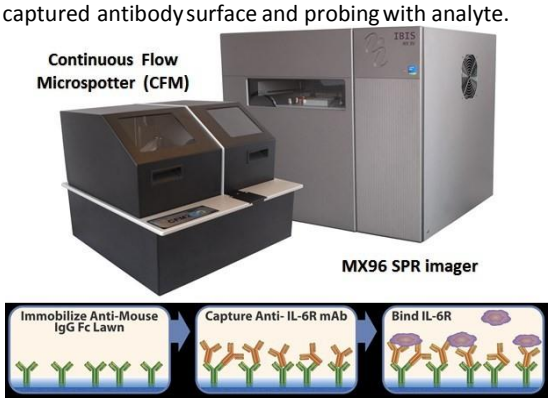

Figure 1. *The Wasatch Microfluidics CFM and IBIS MX96 SPR imager. Shown below is the experimental workflow for an antibody capture kinetics screening strategy of IL-6R against 96 anti-IL-6R mAb sensor spot positions.*

First, a SensEye COOH-G chip was docked in the MX96 instrument primed with 10mM sodium acetate pH 5.0, 0.01% Tween-20 running buffer. Activation of the chip surface was achieved by injection of premixed 1.2mM EDC: 0.3mM sulfo-NHS for 5 min. Goat anti-mouse IgG Fc antibody (Jackson Immuno Research #115-005-071) was diluted in running buffer and was cycled across the activated surface using bidirectional flow for 4 min, based on conditions determined in earlier studies as suitable for generating a low density lawn. Remaining active sites were quenched by 5 min of contact with 0.5M ethanolamine pH 8.5.

Capture of anti-IL-6R mAb

After generation of the anti-mouse IgG Fc mAb lawn on the IBIS, the sensor chip was loaded into the Continuous Flow Microspotter (CFM) primed with 1X PBS pH 7.4, 0.01% Tween-20 system buffer. Anti- human IL-6R mAb (R&D Systems #MAB227) was prepared as a 3-fold serial dilution from 33 to 0.02µg/ml in system buffer, yielding 8 concentrations in total. Contact times for capture of 70ul of anti-IL-6R mAb were either 1, 3, 9, or 27 min, with sample being returned to the source plate after capture.

Binding to IL-6R to captured surface

Following capture of 96 individual anti-IL-6R mAb spots on the anti-mouse IgG lawn, the sensor chip was re-docked in the MX96 and primed with 1X PBS pH 7.4, 0.01% Tween-20 running buffer. IL-6R (R&D Systems #227-SR/CF) was prepared from 33 to 0.05nM in running buffer as a 3-fold serial dilution.
IL-6R injections at 150ul were cycled across the chip surface for 7 min followed by dissociation in running buffer for 40min, both at 45ul/min. No regeneration was performed between analyte injections and reference data were collected on anti-mouse IgG Fc interspots.

FPK data analysis

Kinetics data were first processed using IBIS SPRint software for calibration of sensor signals and interspot reference subtraction, then exported to Scrubber for kinetics fitting. Using a modified version of Scrubber specifically designed for arrayed data sets, the sensorgrams were zeroed, cropped, aligned, buffer injection referenced, and then globally fitted to a 1:1 binding model. As this experiment did not include regeneration between analyte injections, injection start time was selected as a floating parameter in order to fit association profiles back to a theoretical baseline origin.

Biacore 2000 kinetics

A CM5 chip was docked in a Biacore 2000 with 1X PBS -T pH 7.4, 0.01% Tween-20 running buffer. Activation of the surface was achieved using a mixture of 1.2mM EDC: 0.3mM sulfo-NHS, followed by amine coupling 5000 RU goat anti-mouse IgG Fc diluted in 10mM sodium acetate pH 5.0, 0.01% Tween 20. The surface was quenched using 0.5M ethanolamine pH 8.5. Anti-human IL-6R mAb diluted in running buffer and captured at 100 RU on the chip surface, followed by injection of 33-0.05nM IL-6R. In contrast to the experiment conducted on the MX96, following analyte binding and dissociation, the surface was regenerated with 10mM glycine pH 2.5. Data were globally fitted in Scrubber using a 1:1 model.

Results

Sensor chip preparation and kinetics fitting

In order to generate a uniform "lawn" surface, anti- mouse IgG Fc was amine coupled across the entire COOH-G chip using the MX96, with sensorgrams for actively recorded spots shown in Figure 2.

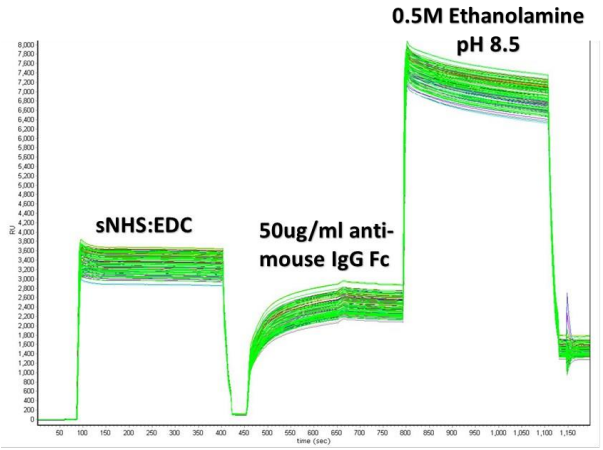

Figure 2. *MX96 sensorgrams for amine coupling of anti-mouse IgG Fc lawn.*

After capture of anti-IL-6R mAb using the CFM, the chip was re-docked in the MX96 and injections of increasing analyte concentrations were monitored in parallel across all 96 ligand positions, depicted in Figure 3. Note the absence of regeneration cycles between analyte injections.

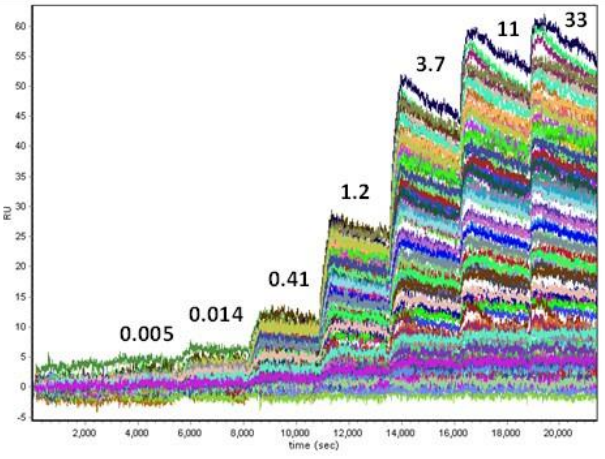

Figure 3. *MX96 sensorgram overlays of ascending concentrations of IL-6R injected across anti-IL-6R array. IL-6R concentrations (nM) are noted above each injection.*

In Figure 4, tiled views of 1:1 Scrubber sensorgram fits for each of the 96 ligand spots generated using the combination of the MX96 and CFM are displayed. On the left-hand side of the figure are the varied CFM capture times for pairs of rows, each having 8 descending ligand densities captured in triplicate.

Figure 4. *Tiled view of fitted sensorgrams for all sensor locations using version of Scrubber modified for array- based data. Noted are anti-IL-6R mAb capture times from the CFM.*

Uniformity and sensitivity of kinetic responses

Overall analyte binding responses are consistent with the varied capture ligand concentrations and contact times. As an example, in Figure 5 the IL-6R response against anti-IL-6R mAb captured at 0.14µg/ml for 1 min is the below the limit for accurate fitting due to poor signal to noise. By increasing the contact time to 9 min while using the same concentration of anti-IL-6R mAb, the sensorgrams were readily fitted. Furthermore, by generating a lower density amine coupled anti-mouse IgG Fc lawn, an upper bound is imposed on levels of anti-IL-6R mAb that can be captured. This enables screening of antibody samples with highly varied concentrations, as capture levels will rapidly plateau for higher concentration samples, while lower concentration samples can gradually reach kinetically useful levels by extending capture contact times in the CFM.

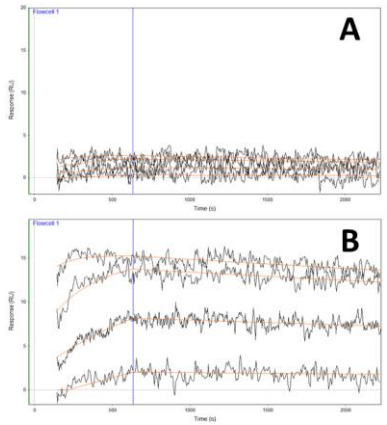

Figure 5. *Comparison of SPR signals for IL-6R binding to 0.14mg/ml anti-IL-6R mAb captured at 1 min (A) or 9 min (B). Extending contact time to 9 min in CFM enabled measurable signal while consuming no additional antibody.*

The relationship of affinity as a function of changing contact times and ligand concentration for anti-IL-6R mAb is shown in Figure 6.

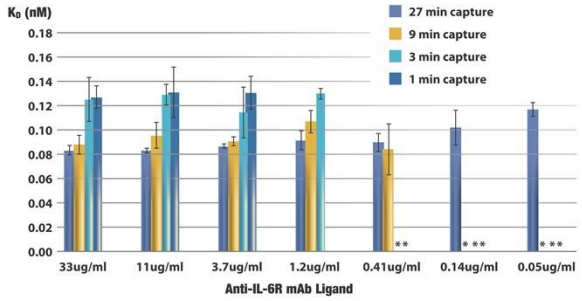

Figure 6. *Effect of ligand antibody concentration and contact time on affinity. Calculated affinity remains stable across a wide range of concentrations and is largely dependent on the capture contact time, with short contact times limiting the range of kinetically useful data.*

Data are averages of triplicate measures on the sensor surfaces and, as the error bars indicate, kinetic values show good correlation when spotted at different positions on the sensor surface, demonstrating the overall reproducibility of these printed arrays. At concentrations of the ligand mAb between 33 and 3.7µg/ml, calculated affinity values edged slightly higher with reduced contact times, but remained within an approximately 30% range. Below 3.7µg/ml, poor signal to noise begins to exclude samples with lower contact times. Notably kinetic fitting and subsequent calculation of affinity is obtainable for ligand mAb captured at 0.05µg/ml by extending the contact time to 27 min, highlighted in Figure 7.

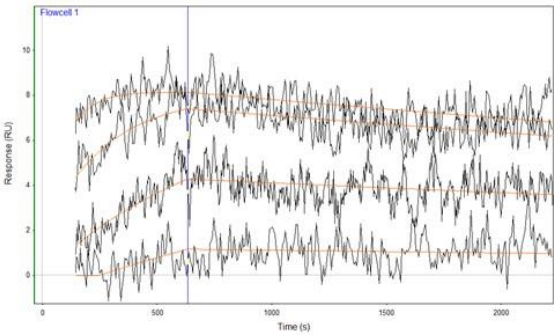

Figure 7. *Fitted sensorgrams for 33-0.05nm IL-6R binding to 0.05µg/ml anti-IL-6R antibody surface captured for 27 min. Despite the increased signal to noise the calculated affinity, ~12nM, correlated well with the ~9nM affinity observed for the same sample at captured at higher concentrations.*

Comparison of First-Pass Kinetics™ screening approach to traditional SPR

For comparison of results from the First-Pass Kinetics™ approach to a lower throughput SPR strategy, the same sample system was analyzed on a Biacore 2000, with the exception that the Biacore chip surface was regenerated between analyte injections.

Figure 8 shows the double-reference subtracted sensorgrams for a serial dilution of IL-6R analyte run in duplicate with 1:1 global fits applied along with calculated kinetics parameters. The association rate ($6.08e^{5}$ s^{-1}) obtained on the Biacore is very similar to the average measured using the First-Pass Kinetics™ approach ($7.76e^{5}$ s^{-1}) in which no regeneration was performed between analyte injections and association rate start times were floated during fitting. For the rate of dissociation, the Biacore system shows about a 6.5-fold slower rate of dissociation ($1.28e^{-5}$ M^{-1} s^{-1}) compared with average values obtained on the MX96 ($8.42e^{-5}$ M^{-1} s^{-1}). This indicates that dissimilarities in k_d most likely drive the tighter affinity observed in Biacore and could be a function of modest differences in baseline drift and/ or reference subtraction. The faster dissociation rates seen in the MX96 also indicate that re-binding was likely not significant in this platform. Although calculated affinities between the MX96 and Average, they remain in reasonably good agreement given the superior number of unique measures the First-Pass Kinetics™ approach affords and overall show good reproducibility across the sensor surface.

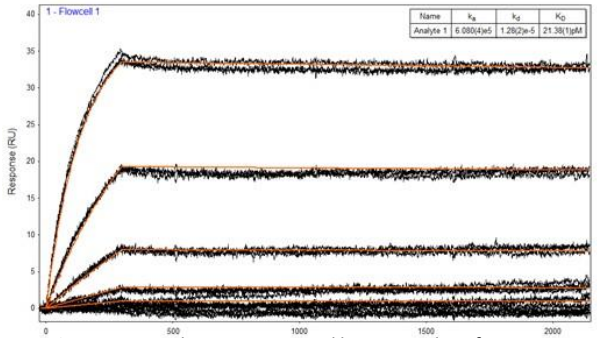

Figure 8. *Fitted sensorgrams and kinetics values for IL-6R binding anti-IL-6R on a Biacore 2000.*

Visualization of mAb kinetic trends using the First-Pass Kinetics™ software tool

The relationships of k_a, k_d, and K_D values derived from Scrubber were visualized using the First-Pass Kinetics™ Tool developed by Wasatch Microfluidics. This application generates interactive iso-affinity plots with options to rapidly select and highlight subgroups of mAbs based on kinetic profiles. In Figure 9 a screenshot of the software user interface is shown. Plotted are the iso-affinities for IL-6R and anti-IL-6R mAb measured using the MX96 and CFM as well as with the Biacore 2000. Also included in the plot are kinetics values for 56 mAbs unrelated to this study, but nonetheless included to demonstrate the breadth of affinities that can be simultaneously measured in the system. Owing to the previously discussed reproducibility of kinetics, the IL-6R mAbs are tightly clustered and in reasonably close proximity to the same system run on the Biacore 2000.

An additional feature of the First-Pass Kinetics ™ tool that aids in rapid visual assessment of larger datasets is the size of the plotted circles which are indicative of the potential data quality issues. Small circles flag samples with Rmax < 10 or Rmax > 500 and a ratio of ResSd/Rmax > 0.1, particularly important where binding responses and signal to noise become non-ideal for kinetics fitting. Rmax and ResSd are both exported components from kinetics analysis in Scrubber. These small circles flag data that may require closer inspection from the analyst.

Figure 9. *View of First-Pass Kinetics™ software interface showing iso-affinity plot for IL-6R against anti-IL-6R mAbs (blue dots circled in red). Plotted in green and circled in red is the k_a and k_d for the same system run on a Biacore. Also included is an unrelated antigen-antibody kinetics experiment using a similar workflow for 56 mAbs (yellow dots), showing the diversity of kinetics that can be simultaneously screened and evaluated.*

Conclusion

Demonstrated here is a strategy for high throughput SPRi analysis of 96 unique binding interactions to determine k_a, k_d, and K_D, using both an advanced hardware platform for screening protein arrays as well as software tools effectively manage these more complex data sets. These studies were completed over the course of a typical work day on a single sensor chip using minimal quantities of IL-6R antibody and antigen. The ability to extend capture times with no change in ligand consumption was key to obtaining usable kinetic signals when antibody concentrations were in the mid ng/ml range, while still allowing for fitting of samples with concentrations more than two orders of magnitude higher. Good reproducibility was observed for replicate spots across the sensor chip and measured affinity was within approximately 5-fold of a traditional SPR system. This simultaneous monitoring of detailed kinetics in real-time utilizing an array of diverse ligand parameters offers a number of unique experimental designs to accelerate kinetic screening strategies, where currently throughput is substantially limited. Taken together with other biophysical properties such as epitope and stability, the First-Pass Kinetics™ approach provides a wealth of information for comprehensively screening and ranking candidate antibodies.

References

[1] G. C. Barnard, M. D. Hougland, and Y. Rajendra, “High-throughput mAb expression and purification platform based on transient CHO.,” *Biotechnol. Prog.*, Nov. 2014. doi: 10.1002/btpr.2012

[2] F. Ylera, S. Harth, D. Waldherr, C. Frisch, and A. Knappik, “Off-rate screening for selection of high-affinity antidrug antibodies.,” *Anal. Biochem.*, Oct. 2013. doi: 10.1016/j.ab.2013.07.025