APPLICATION NOTE

nicoya

Characterization of Fc Gamma Receptor and IgG Interactions using Alto: Comparison to BLI

Summary

Label-free analysis of biomolecular interactions is critical in the development of biologics, but current analytical instruments are not equipped to meet the needs of the modern biologics researcher. They require substantial amounts of sample and years of experience to produce high-quality data. Alto™, Nicoya's Digital SPR™ platform, solves these problems through the use of digital microfluidics and integrated LSPR sensors.

This application note demonstrates the application of Alto for measuring biomolecular interactions and generating data that is comparable to biolayer interferometry (BLI). Alto requires only a fraction of sample and hands-on time to obtain affinity and kinetics data that is highly-agreeable with a standard BLI platform, making it an extremely efficient platform for biologics discovery and development.

Introduction

Label-free analytical methods are used in target molecule identification to lead selection and optimization during the therapeutic discovery workflow. Kinetic parameters collected by label-free analytical instruments are essential for understanding the mode of action of the candidate therapeutic against its target and its advancement in the discovery pipeline.

Label-Free Analytical Methods

Biolayer-Interferometry (BLI)

BLI platforms use an interference pattern of white reflected light from immobilized protein on the sensor tip to collect data. In these systems, an internal reference layer is used. During the experiment a sensor tip is dipped into the samples to perform the experiment. While this technology eliminates the need for complex networks of pumps, valves and flow cells, it still requires a minimum of 40-220 µL of sample per concentration, depending on the plate type used. It is also limited in measurement time due to evaporation and often exhibits significant mass transport effects, all of which limits the affinity and kinetic range it can accurately operate within.

Digital Surface Plasmon Resonance (SPR)

The Alto platform uses a digital microfluidics-powered localized SPR (LSPR) system for label-free characterization of biomolecule interactions. In Alto, all experimental reagents such as samples and buffers are loaded and self-contained into a disposable cartridge. Voltage is applied across electrodes to dispense, move, mix, and conduct dilution series on 350 nL droplets inside the cartridge. When the experiment is complete, the cartridge is easily discarded and data can be readily analyzed with the click of a button. All together, Alto requires minimal training to use, is compatible with crude samples, and requires only 2 µL sample volumes for full kinetic characterization across a wide range of affinity and kinetics. A comparison of Alto to BLI is summarized in Table 1.

Table 1: Comparison of Alto Digital SPR and BLI.

	Alto	BLI
Technology	Localized Surface Plasmon Resonance (LSPR)	Bio-Layer Interferometry
Sample Processing	Digital microfluidics (DMF)	Dip and read
Sample Volume	2 µL*	40-220 µL**
Serial Dilutions	Automated on cartridge	Manual
Unattended Run Time	24+ hours	Up to 12 hours
Hands-on Time	<30 mins	60+ mins

**total for all concentrations*
***per individual concentration*

Application of SPR in Understanding Fc Receptors

Fc Gamma Receptors (FcγR)

A Fc receptor is a protein that belongs to the immunoglobulin superfamily and contributes to the protective functions of the immune system. Its name is derived from its binding specificity for a part of an antibody known as the Fc (fragment crystallizable) region[1]. Fc receptors bind to antibodies on the surface of infected cells or invading pathogens. Their activity triggers phagocytic or cytotoxic cells to destroy microbes or infected cells by antibody-mediated phagocytosis or antibody-dependent cell-mediated cytotoxicity[1,2]. There are many groups of Fc receptors based on the type of antibody they recognize. One type of Fc receptor is the Fc gamma receptor (FcγR) which is a cell-surface receptor that binds to immunoglobulin G (IgG)[2].

There are 3 different classes of human FcγR (FcγRI, FcγRII a & b, and FcγRIII a & b) that have the ability to activate or suppress the immune response[2]. FcγRIa, FcγRIIa, FcγRIIIa and FcγRIIIb are activating via the immunoreceptor tyrosine-based activation motif and FcγRIIb is inhibitory via signaling through the immunoreceptor tyrosine-based inhibitory motif[2,3]. Likewise, mice have receptors that are orthologs to the human FcγRs, these consists of 3 activating receptors (FcγRIa, FcγRIII and FcγRIV) and one inhibitory FcγR (FcγRIIb)[4]. Each FcγR exhibits different binding specificity and affinity towards the various IgG subclasses[4,5]. It is important to get a better understanding of the molecular mechanisms involved in FcγR function as their dysregulation is an important factor in several autoimmune diseases.

Characterizing Antibody Binding to FcγRs with Digital SPR

SPR is well-suited for characterizing FcγRs. Advantages of SPR include:

- No need for additional labels, eliminating the introduction of another irrelevant binding event to account for and design around.
- Acquisition of real-time binding data that enables deeper insight into the binding kinetics of antibody subclasses to Fc gamma receptors.

Alto has a number of features that make it ideal for SPR characterization of Fc gamma receptors and their antibody targets, compared to other label-free tools such as BLI.

With the ability to discreetly control nano-liter sized droplets of each ligand and analyte, binding assays are simple to implement and provide novel insights into therapeutic performance, while reducing consumption of precious samples by up to 200X when compared to traditional SPR and BLI systems. In addition, Alto's 16 independent channels provide the ability to simultaneously analyze multiple targets in many different assay formats, while significantly reducing hands-on time with complete assay automation.

In this application note, we demonstrate the use of Alto in measuring binding affinities and kinetics of mouse Fc gamma receptor I (FcγRI) and the mouse antibody IgG2A through several experimental approaches. Results are then compared to those obtained with BLI technology.

Materials & Equipment

- Nicoya Alto 16-Channel Instrument
- Alto 16-Channel Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Carboxyl Surfacing Kit (ALTO-R-CBX-SURF)
- Nicoya Streptavidin Kit (ALTO-R-STV-KIT)
- Ligand and analyte:
  - Biotinylated Mouse Fc gamma RI / CD64 Protein, His, Avitag™ (Acro Biosystems, Cat: CD4-M82E7)
  - Mouse IgG2A Isotype Control (R&D Systems, Cat: MAB003)

Method

Full method is detailed in the Appendix.

Results & Discussion

Data Quality

The capture of the bt-FcγRI ligand on the streptavidin surface on Alto was optimized to obtain a low ligand density while allowing for detection of all analyte concentrations sampled. Kinetic values were calculated

based on the sensorgrams generated by the Alto and the BLI instruments. The Alto kinetics were calculated with a 1:1 Langmuir fit which resulted in an excellent fit of the data, adding a MTL correction did not have a significant change in the kinetic values (Figure 1). The BLI data was fit with both a 1:1 and a 1:1 diffusion corrected (MTL) fit model, with the diffusion corrected model showing a significantly improved fit (Figure 2). This implies a mass-transport contribution to the BLI data. Comparison of the kinetic values obtained by the Alto and BLI MCK assays revealed similar results, as shown in Table 2. Visual comparison of the MCK data sets obtained with Alto and BLI showed comparable binding curves and fit overlays for each analyte concentration. In summary, Alto was able to generate kinetic data results and binding curves that compare very well with the BLI instrument, without significant contributions from mass transport.

Ease of Use

The experiment described above was conducted on Alto and the BLI technology based instrument to compare the performance of the two devices as summarized in Figure 3. This experiment required 28 mins of operator time with Alto versus 66 mins with the BLI instrument. Side by side experimental workflow comparison revealed that 25 minutes of operator time was saved in preparation of sample dilutions with Alto's on-cartridge dilutions. Furthermore, Alto's intuitive software, prepared templates and automated analysis tool has saved an additional 27 mins of operator time. Overall, when compared to the BLI technology, Alto is at least 2X faster, saving approximately 38 mins of hands on time during this experiment. In addition to the savings in operator time, the Alto used a total of 2 µL of sample, whereas the BLI technology based instrument required 900 µL of sample.

Table 2: Comparison of the kinetic data parameters calculated from the sensorgrams collected by Alto and BLI experiments.

	Fit Model	K_t (RU/(M*s))	k_a (1/(M*s))	k_d (1/s)	K_D (M)
Alto	1:1	N/A	8.81E+05	2.69E-03	4.50E-09
BLI	1:1	N/A	2.65E+05	2.48E-03	9.36E-09
BLI	1:1 with MTL	4.40E5	8.62E+05	6.61E-03	7.67E-09

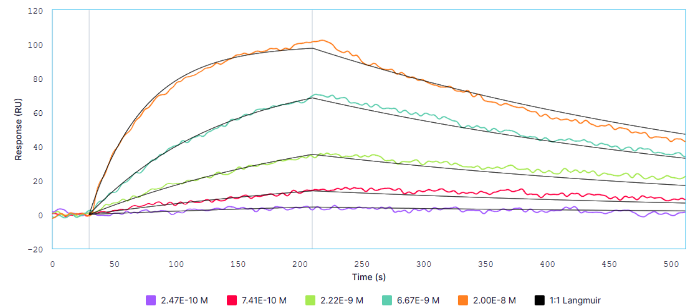

Figure 1: *Kinetics of IgG2A (analyte) binding to captured biotin-FcγRI (ligand) on Alto using multi-cycle kinetics (MCK). Analyte was titrated from 1.23 nM to 100 nM. Black curve represents the Langmuir 1:1 binding fit.*

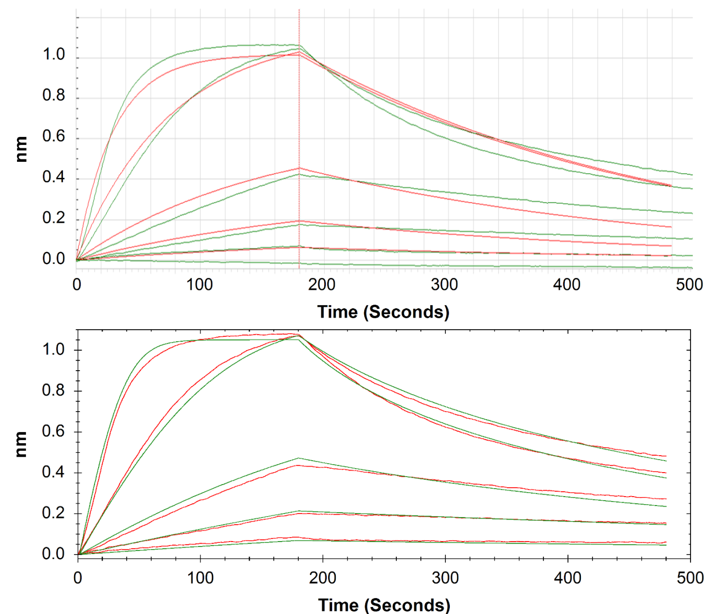

Figure 2: *Multi-cycle kinetics of IgG2A (analyte) binding to captured biotin-FcγRI (ligand) on BLI. Analyte was titrated from 1.23 nM to 100 nM. Green curve in the top plot represents the Langmuir 1:1 binding fit obtained by the CRO. Green curve in the bottom plot represents the Langmuir 1:1 Diffusion Corrected binding fit obtained in Tracedrawer.*

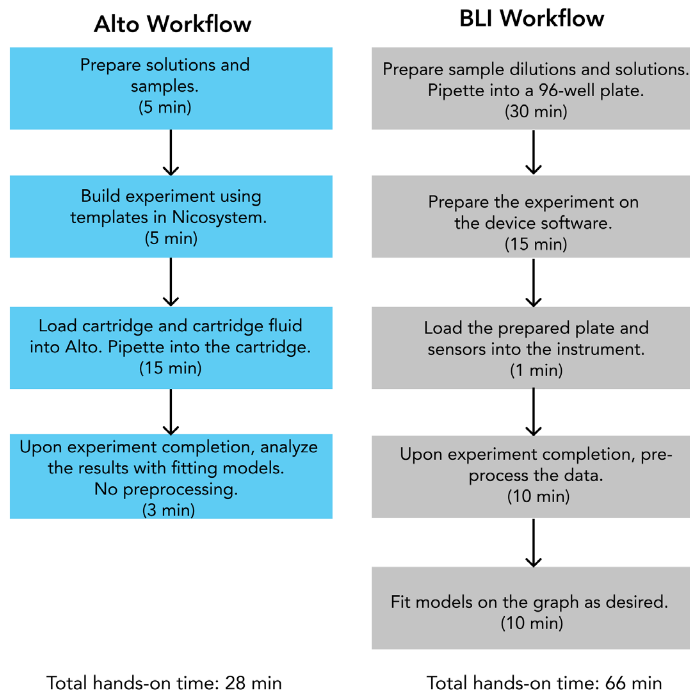

Figure 3: *Flow chart of Alto vs BLI workflow with step time summary. This figure summarizes side by side user workflow steps executed from sample preparation to data analysis. Overall, the same experiment required 28 minutes of hands-on operator time with Alto, compared to 66 minutes with the BLI instrument.*

Conclusion

When compared to the conventional SPR or BLI systems, the Alto platform demonstrated equivalent accuracy for obtaining kinetic measurements for k_a, k_d, and K_D. All aspects of the experiment were automated by Alto using DMF technology, allowing all analysis to be conducted on a single cartridge with just a fraction of typical sample requirements, while requiring just 30 minutes of hand-on time with the instrument. The Alto workflow significantly decreases the burden of preparing large experimental production batches for biologic screens and accelerates time to discovery. The resulting data highlights Alto's ability to provide high-quality data while reducing time to answer, proving its ability to accelerate the characterization of binding specificity and affinity of Fc receptors towards their various IgG subclasses.

References

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Appendix

Method

This label-free SPR assay was performed using Alto. Alto uses a cartridge-based, gold nanostructure sensor with 16 channels (8 reference channels and 8 active channels). The experimental method was designed remotely using Alto's cloud-based user portal, the Nicosystem™, which was uploaded to the instrument with a click of a button.

Alto

First, a 16-Channel Carboxyl Cartridge was loaded into Alto followed by dispensing of the cartridge fluid into the cartridge. All the reagents were pipetted into the cartridge wells following software-directed prompts. Since Alto is an automated SPR instrument, only a single top concentration of each analyte sample was pipetted.

After all the reagents were loaded into the cartridge, the experiment was initiated by selecting the "Run Method" command on Alto. All subsequent steps were automated by Alto.

In all experiments, normalization of sensors with high and low RI droplets was first performed. The sensors were then cleaned with 10 mM HCl for 60 s, followed by a 5 minute activation of the 16 carboxyl sensors with a drop consisting of a 1:1 ratio of 25 mM EDC/NHS mixed on cartridge. The capture molecule, streptavidin (60 µg/mL) in 10 mM sodium acetate pH 5.0, was immobilized onto all 16 sensors for 5 mins, and all sensors were subsequently blocked with 1 M ethanolamine for 5 mins to quench the remaining active carboxyl groups. The biotinylated FcγRI ligand at 2.5 µg/mL in PBS-T was passed over the 8 active channels for 5 mins and captured by the streptavidin molecule.

For the IgG2A analyte, 2 µL of 300 nM solution was loaded into each analyte well, and the mixing of five 3-fold serial dilutions per analyte was automated by Alto. The concentrations of IgG2A used for binding were 1.23 nM, 3.70 nM, 11.1 nM, 33.3 nM and 100 nM. Replicates of the IgG2A analyte were introduced in a multi-cycle kinetics (MCK) format. The MCK format used an association time of 120 s, with a dissociation time of 600 s after each association. After each dissociation, the analyte was fully dissociated.

BLI

The BLI experiment and analysis was outsourced to a CRO. The protocol performed was as follows. The biotinylated FcγRI protein at 5 µg/ml was captured using a Streptavidin coated (SSA) dip and read biosensors for 60 s. Parallel ligand-free reference sensors were used. The probes were dipped into wells with analyte, mouse IgG2A control isotype antibody (150 kDa) at 6.25 nM for scouting and starting at 100 nM, followed by 3 fold serial dilution for full kinetics. PBS with 0.1% Tween 20 used for analyte dilutions and baseline buffers. Association rate (on rate) of the IgG2A was measured. The probes were dipped into the assay buffer for 600 s. (PBST) and the dissociation rate (off rate) was measured. The K_D was determined from the obtained k_a and k_d using 1:1 local fit analysis on both the Fortebio software (fit provided by CRO) and using TraceDrawer, with MTL correction applied.