APPLICATION NOTE

nicoya

Designing label-free biosensor assays for Fc receptor binding analysis: Meeting research challenges

Abstract

Understanding the binding and kinetics of Fc gamma receptors (FcγR) to monoclonal antibodies is critical for advancing the development of targeted therapies aimed at modulating immune responses effectively. These interactions can greatly impact therapeutics safety and efficacy, making efforts to analyze and enhance Fc interactions with FcγRs an important aspect of the development of biotherapeutics.

When analyzing Fc receptor binding using label-free biosensors, the complexity involved in assay development and optimization places hurdles in accessing high-quality data. Often, a high level of expertise is needed to develop optimal assay methods, high volume of expensive antigens may be needed, and time to results can be long.

In this application note, we demonstrate how the Alto™ Digital SPR™ system offers a superior innovation in label-free biosensors for the measurement of binding affinities and kinetics of antibodies to different classes of FcγRs, highlight best practices for various assay formats and capture surfaces, and describe assay optimization and data analysis.

Application of SPR in understanding Fc receptors

Fc gamma receptors (FcγR)

Fc receptors are a family of membrane-bound glycoproteins that belong to the immunoglobulin superfamily and contribute to the protective functions of the immune system. The name is derived from the receptors binding specificity for the antibody Fc (fragment crystallizable) region.[1] Fc receptors bind to antibodies on the surface of infected cells or invading pathogens (Figure 1). Their activity triggers phagocytic or cytotoxic cells to destroy microbes or infected cells by antibody-mediated phagocytosis or antibody-dependent cellular cytotoxicity (ADCC).[1, 2] Fc receptors are grouped based on the type of antibody they recognize. One such type is the Fc gamma receptor (FcγR) that is a cell-surface receptor that binds to immunoglobulin G (IgG).[2]

There are 3 different classes of human Fc gamma receptors, namely, FcγRI, FcγRII A & B, and FcγRIII A & B, each of which have the ability to activate or suppress immune responses.[2] FcγRI, FcγRIIA, FcγRIIIA and FcγRIIIB activate immune responses via the immunoreceptor tyrosine-based

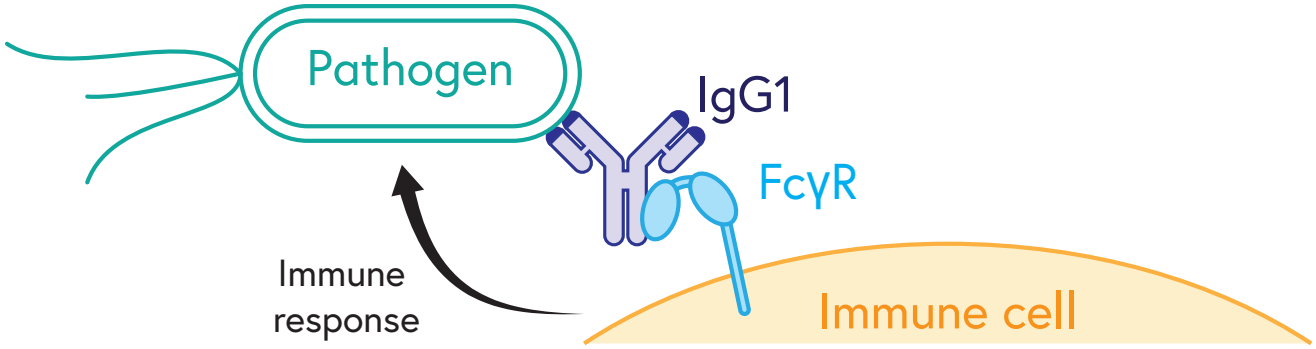

Figure 1: *Illustration of how Fc gamma receptors (FcγRs) bind antibodies to trigger an immune response.*

activation motif while FcγRIIb inhibits immune responses via signaling through the immunoreceptor tyrosine-based inhibitory motif.[2, 3] Like humans, mice have orthologous Fc receptor classes, consisting of 3 activating (FcγRI, FcγRIII and FcγRIV) and one inhibitory receptor class (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 dysregulation of FcγR activity has been implicated in various medical conditions, including autoimmune diseases, cancer, and infectious diseases.

Understanding the binding and kinetics of FcγRs to monoclonal antibodies is critical for advancing the development of targeted therapies aimed at modulating immune responses effectively. In this application note, we demonstrate the use of Alto in measuring binding affinities and kinetics of various classes of FcγRs through several experimental approaches. We describe best practices for various assay formats and capture surfaces, including assay optimization and analysis.

Characterizing antibody binding to FcγRs with Alto Digital SPR

SPR is well-suited for characterizing FcγRs due to its ability to measure the binding affinity between receptors and IgG molecules or therapeutic antibodies. In addition to affinity, SPR provides detailed kinetic parameters, such as the association rate constant (k_{on}) and dissociation rate constant (k_{off}), revealing how quickly an antibody binds to or dissociates from the Fc receptor.

Typical SPR assay design and development must consider some assay complexities. Potential interference from non-specific binding to the sensor surface, potential alterations to the receptor's conformation upon immobilization, difficulty in distinguishing subtle binding affinity differences, mass transport limitations, and the need for careful control experiments to mitigate false positives due to surface effects can all impact the accuracy and reliability of the binding data. Effective assay development and optimization can be time, labor, and reagent consuming. An assay platform that helps rapidly optimize assay conditions and obtain high data quality can significantly accelerate time to results.

Alto is the world's first SPR platform to integrate digital microfluidics (DMF) with nanotechnology-based biosensors. Alto has a number of features that make it ideal for SPR characterization of Fc gamma receptors and their antibody targets. 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. Furthermore, Alto's 16 independent channels provide the ability to simultaneously analyze multiple targets in many different assay formats, significantly reducing hands-on time with complete assay automation and automatically diluted sample preparation. Together these features make Alto an ideal platform to overcome the challenges associated with characterizing FcγR interactions.

Developing an FcγR/IgG binding kinetics assay

Choosing an immobilization strategy

Alto's Carboxyl Cartridge is a disposable cartridge containing 16 carboxyl-functionalized sensors. To perform an SPR experiment on the Alto, one of the binding partners (ligand) is immobilized on the sensor surface, and the other partner (analyte) is passed over the surface. The 16 sensors are arranged into 8 lanes with two sensors per lane, one being an active sensor and the other a reference sensor. The active sensor is equipped with the immobilized ligand, where binding interactions with the analyte occur. In contrast, the reference sensor flows the analyte over its surface without a ligand, capturing a background signal. This background signal is subsequently subtracted from the analyte response measured on the active sensor to ensure accurate results. In this section, the assay orientation, i.e. which of the FcγR or IgG will be immobilized, as well as various immobilization options will be discussed. The choice of an optimal immobilization method depends on the number of FcγRs and IgGs to be tested and availability of FcγRs with affinity tags.

Anti-HIS capture of HIS-tagged FcγR

HIS-tagged FcγRs can be immobilized using Nicoya's Anti-HIS Capture Kit (Figure 2). The Anti-HIS Capture Kit works by amine coupling the anti-HIS antibody to carboxyl sensors of the Alto cartridge. Using Alto's capture kinetics application, users can capture their HIS-tagged FcγRs onto the immobilized antibody and measure kinetics between their IgGs and the captured FcγR. The immobilization of the anti-HIS antibody to the reference channel as well as the active channel reduces the likelihood of non-specific binding of the IgG to the surface.

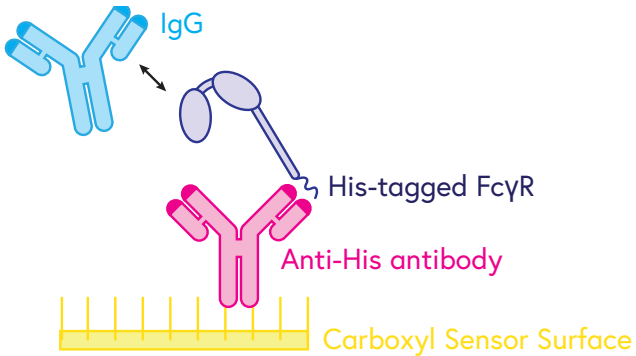

Figure 2: *Schematic representation of an assay using the Anti-HIS Capture Kit.*

Streptavidin capture of biotinylated FcγR

Direct kinetics with streptavidin can be used when analyzing biotinylated FcγRs. In this format, which utilizes the Alto Streptavidin Kit (Figure 3), streptavidin is directly amine coupled to the carboxyl surface. The biotinylated FcγR then irreversibly binds to the streptavidin, effectively immobilizing the ligand on the surface with minimal steric hindrance for IgG binding. The IgG analyte will then be analyzed against the ligand surface. In this format, streptavidin is also immobilized on reference sensors as it is more representative of the active sensor surface and can help reduce non-specific binding. Because the biotin-streptavidin interaction can not be regenerated, the ligand is not reapplied to the surface after each regeneration step. Streptavidin surfaces are extremely robust and have reliable, consistent capture of biotin-tagged molecules.

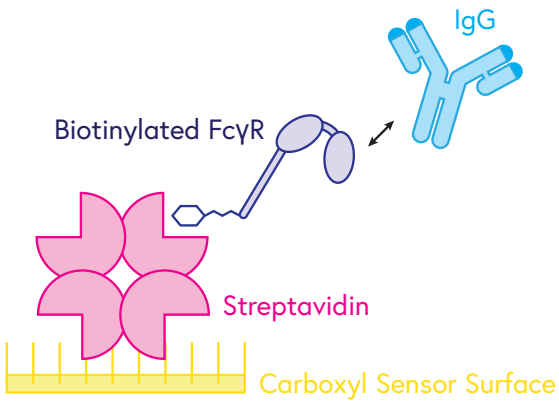

Figure 3: *Schematic representation of an assay using the Streptavidin Kit.*

Protein A capture of the IgG

Nicoya's Protein A Capture Kit (Figure 4) allows users to capture IgGs directionally, allowing the IgG to be used as the ligand and FcγRs as the analyte. The protein A binding site on IgG is distinct from the FcγR binding sites. FcγRs bind to the IgG's Fc at the hinge region of its CH2 domain, while protein A typically binds the CH2-CH3 interface of the IgG Fc domain.[6] This capture method allows for the analysis of many IgGs against many FcγRs as the protein A surface can be readily regenerated.

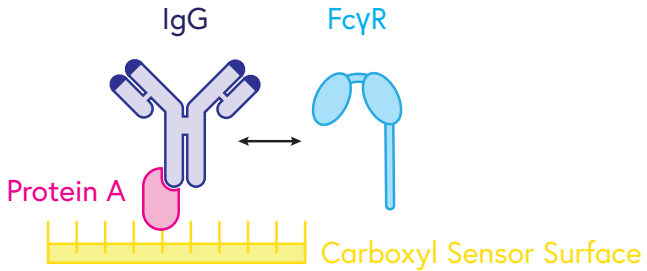

Figure 4: *Schematic representation of an assay using the Protein A Capture Kit.*

Other immobilization strategies

Beyond the ligand capture methods described above, there are a number of other ligand immobilization methods available for FcγRs and IgGs. These include direct immobilization of either FcγR or IgG and immobilizing the IgG by capturing the Fab region. These methods, which can be easily performed on Alto, were not tested here and therefore will not be discussed in detail in this application note.

Optimizing experimental conditions

This section will walk through the assay development steps for determining the optimal experimental conditions for analyzing FcγR and IgG kinetics on the Alto platform. Guidance on ligand loading density, analyte concentration, buffer conditions, and regeneration for obtaining accurate and reliable kinetics and affinities will be discussed. More details on optimizing conditions can be found using Nicoya's eBook: Mastering kinetic binding assays.

Ligand density

For any kinetics assay, the ligand immobilization level can have a big impact on the quality of kinetics determined. Using too low of a concentration risks failure to observe binding of the analyte or having a low signal to noise

ratio. Using too high of a concentration risks introduction of artifacts such as steric hindrance, avidity and mass transport that can make the interpretation of results challenging, as demonstrated in Figure 5.

When analyzing FcγR and IgG kinetics, optimal ligand density will depend on the orientation and immobilization strategy used, but a low ligand density will provide the best results. For capture of the HIS-tagged FcγR using the Anti-HIS Capture Kit it is recommended to use a concentration of 0.5-5 µg/mL of FcγR to obtain about 200-300 RU of binding signal.

For the capture of biotinylated FcγRs using the Streptavidin Kit, it is recommended to use a low concentration between 0.1-2 µg/mL of the FcγR to obtain about 100-200 RU of binding signal.

For capture of IgGs using the Protein A Capture Kit, a higher capture response is recommended to determine optimal kinetics because the IgG has a larger molecular weight than the FcγR. It is recommended to use a concentration of 0.5-10 µg/mL for the IgG ligand to obtain between 500-800 RU of binding signal.

Lastly, if using direct immobilization of the IgG as ligand, a concentration of 2.5-15 µg/mL should be used to provide about 500-1200 RU of binding signal. For direct immobilization it is recommended to perform a ligand immobilization optimization experiment to determine the lowest ligand level that provides an R_{max} between 50-150 RU for the FcγR analyte interaction.

Analyte concentration range

Analyte concentrations should cover a range above and below the K_D of the interaction. If the concentrations are too high or too low relative to the K_D, the curves may be tightly grouped and the K_D value determined experimentally will be skewed too high or low, respectively. An advantage of the Alto cartridge is the ability to test multiple analyte concentrations ranges in a single experiment to help quickly optimize assays. A general guideline is that the highest analyte concentration should be ~10-20 times the K_D. Another benefit of Alto is the automated on-cartridge dilution of analytes. A user can simply load a single concentration of each sample into the cartridge, and Alto will automatically and accurately perform a serial dilution of each sample. For example, if the K_D of the binding pair being tested is 10 nM, it is suggested to use 100 nM as the highest analyte concentration. In this case, a sample of 300 nM would be loaded into the cartridge and Alto would perform five 3x dilutions to create a concentration series of 100, 33, 11, 3.7 and 1.2 nM for the full kinetic analysis. This would put the highest analyte concentration at 10x the K_D and the lowest analyte concentration at 0.1x the K_D, covering an excellent dynamic range for this particular binding pair.

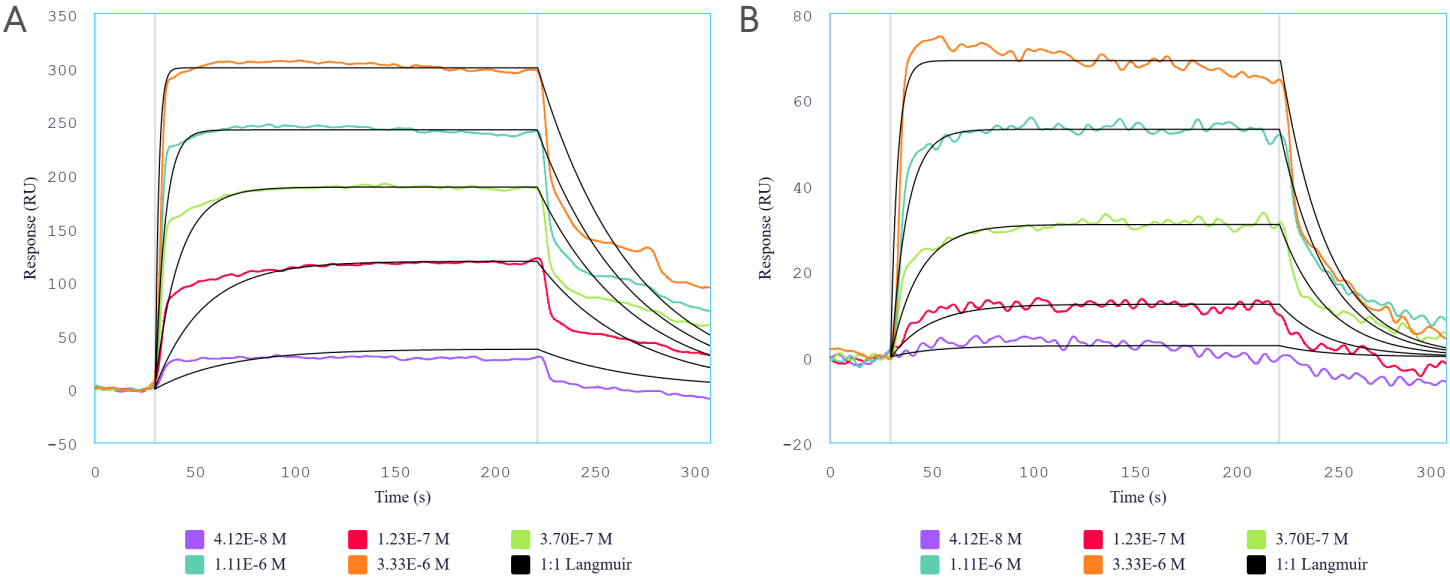

Figure 5: *Kinetic fits for IgG1 binding to HIS-FcγRIIA captured at (A) high and (B) low ligand densities on Nicoya's Anti-HIS Capture Kit. Improved fitting is observed with lower ligand density as demonstrated by a Chi² value of 308 for (A) and 10 for (B) using a 1:1 fit model. Analyte R_{max} is also reduced as a result of lower ligand density.*

For FcγR and IgG kinetics, the high affinity FcγRI interaction typically exhibits a K_D in the low nM range. It is recommended to use a top analyte concentration of 10-100 nM to create a series of 3x diluted analyte samples. The lower affinity FcγRII and III interactions typically exhibit a K_D in the 0.1-10 µM range and thus require a higher analyte concentration range to be used. These interactions typically have fast on rates, resulting in the binding signal reaching equilibrium quickly, therefore it is best to use a shorter contact time for the association phase. The shorter association phase will also prevent any weaker interactions from causing a biphasic curve profile from occurring, allowing for an optimal 1:1 fit of the kinetics data.

Choosing a regeneration solution

Regeneration refers to the application of a solution to the surface to disrupt the binding of the analyte to the ligand, removing the analyte from the sensor. Regeneration is important for optimizing binding kinetics because all of the analyte must be removed from the sensor surface to ensure the same number of ligand binding sites are available before the next concentration of analyte is applied. Removing the bound analyte allows the sensors to be re-used for multiple ligand-analyte interactions. If the natural off-rate of the ligand-analyte complex is fast enough, a regeneration solution may not be needed.

For anti-HIS capture of HIS-tagged FcγRs or Protein A capture of IgGs it is recommended to use a regeneration solution of 10 mM glycine HCl, pH 1.5 to fully remove the captured FcγR-IgG complex from the sensor surface. Following regeneration, a new HIS-tagged FcγR or IgG can be captured on the anti-HIS or Protein A surface, respectively, and a new interaction can be measured.

For the capture of biotinylated FcγRs on the streptavidin surface, the regeneration solution may need to be optimized to determine an ideal solution that will not impact the FcγR binding activity for subsequent IgG binding interactions. For weaker affinity FcγR ligands, which exhibit fast dissociation rates, a regeneration solution may not be needed as long as the IgG fully dissociates in the dissociation phase.

Running an FcγR/IgG binding kinetics assay

Materials

- Alto 16-Channel Instrument with Nicosystem Pro Software (ALTO16)
- Alto 16-Channel CMD Carboxyl Cartridge (KC-CBX-CMD-16)
- Running Buffer: PBS-T (0.1% Tween 20), pH 7.4 (ALTO-R-PBST)
- Carboxyl Surfacing Kit: cleaning, normalization, activation (ALTO-R-CBX-SURF)
- Protein A Capture Kit (ALTO-R-PROA-KIT)
- Streptavidin Kit: (ALTO-R-STV-KIT)
- Anti-HIS Capture Kit: (ALTO-R-HIS-KIT)
- Immobilization Buffer: 10 mM Sodium Acetate Buffer, pH 5.5 (ALTO-R-IMB-5.5)
- Regeneration Solution: 10 mM Glycine-HCl, pH 1.5 (ALTO-R-GLYHCl-1.5)
- Analytes: Tocilizumab (Selleckchem, Cat # A2012), Bevacizumab (Anti-VEGF (RO) IgG1 Antibody) (Sino Biological Cat # 68068-H001)
- Ligands: His-CD16a(176F) Cat# 10389-H08H, biotin-CD16a(176F) Cat# 10389-H27H-B, His-CD16a(176V) Cat# 10389-H08H1, biotin-CD16a(176V) Cat# 10389-H27H1-B, His-CD16b(NA1) Cat# 11046-H08H1, His-CD16b(NA2) Cat# 11046-H08H, biotin-CD16b(NA2) Cat# 11046-H27H-B, His-CD32a(167R) Cat# 10374-H08H, His-CD32a(167H) Cat# 10374-H08H1, biotin-CD32b Cat# 10259-H27H-B, His-CD64 Cat# 10256-H08H. All FcγR products were supplied by Sino Biological.

Methods

Experiment design

The experimental setup was completed remotely on Alto's Nicosystem™ User Portal, followed by run initiation on the instrument:

1. From a laptop, the experiment was designed and saved in the Nicosystem.
2. On the instrument, the designed method was selected to launch Alto's on-screen setup guide.
3. An Alto 16-Channel Carboxyl Cartridge was placed in the instrument, and samples were loaded into the cartridge following the experiment setup guide.
4. The experiment was initiated on the Alto device by selecting "Run Method".

Assay protocols

Capture Kinetics with Anti-HIS Capture Kit

The following steps were completed automatically by Alto with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 25 mM EDC/NHS for 300 s.
4. 10 µg/ml Anti-HIS antibody in 10 mM sodium acetate, pH 4.0 was immobilized on all sensors for 900 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. 5 µg/mL samples of HIS-tagged FcγR ligands in the running buffer were introduced to each even-numbered active sensor for 300 s.
7. Alto executed five automated Tocilizumab serial dilutions on the cartridge. For example, a Tocilizumab sample diluted from 30 µM stock produces 123 nM, 370 nM, 1.11 µM, 3.33 µM, and 10 µM solutions in the running buffer. Different stock concentrations of Tocilizumab are used for each FcγR ligand due to varying affinities.
8. The lowest Tocilizumab concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
9. Step 6 and 8 was repeated for the remaining four Tocilizumab analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Capture Kinetics with Protein A Capture Kit

The following steps were completed automatically by Alto with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 25 mM EDC/NHS for 300 s.
4. The Protein A from the Protein A Capture Kit diluted in 10 mM Sodium Acetate, pH 5.0 was immobilized onto all sensors for 300 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. 10 µg/mL samples of Bevacizumab in the running buffer were introduced to each even-numbered active sensor for 300 s.
7. Alto executed five automated FcγR serial dilutions on the cartridge. For example, an FcγR sample diluted from a 300 nM stock produces 1.23 nM, 3.70 nM, 11.1 nM, 33.3 nM, and 100 nM solutions in the running buffer. Different stock concentrations of each FcγR are used due to varying affinities.
8. The lowest FcγR concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
9. Step 6 and 8 was repeated for the remaining four FcγR analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

Direct Kinetics with Streptavidin Kit

The following steps were completed automatically by Alto with no operator supervision.

1. Carboxyl sensors were normalized with normalization solutions.
2. Carboxyl sensors were primed with 10 mM HCl for 60 s.
3. Carboxyl sensors were activated with 200mM EDC/NHS for 600 s.
4. The streptavidin from the Streptavidin Kit diluted in 10 mM Sodium Acetate, pH 5.0 was immobilized onto all sensors for 300 s.
5. All sensors were blocked with the 1 M ethanolamine for 300 s to quench any remaining active carboxyl groups.
6. 0.1-2 µg/mL samples of biotinylated FcγR ligands in the running buffer were introduced to each even-numbered active sensor for 300 s.
7. Alto executed five automated Bevacizumab serial dilutions on the cartridge. For example, a Bevacizumab sample diluted from a 600 nM stock produces 2.47 nM, 6.41 nM, 22.2 nM, 66.6 nM, and 200 nM solutions in the running buffer. Different stock concentrations of Bevacizumab are used for each FcγR ligand due to varying affinities.
8. The lowest Bevacizumab concentration was exposed to each sensor for 180 s, followed by dissociation in the running buffer for 600 s, and a 60 s regeneration step with 10 mM glycine-HCl, pH 1.5.
9. Step 8 was repeated for the remaining four Bevacizumab analyte concentrations, which constitutes a full multi-cycle kinetics (MCK) round.

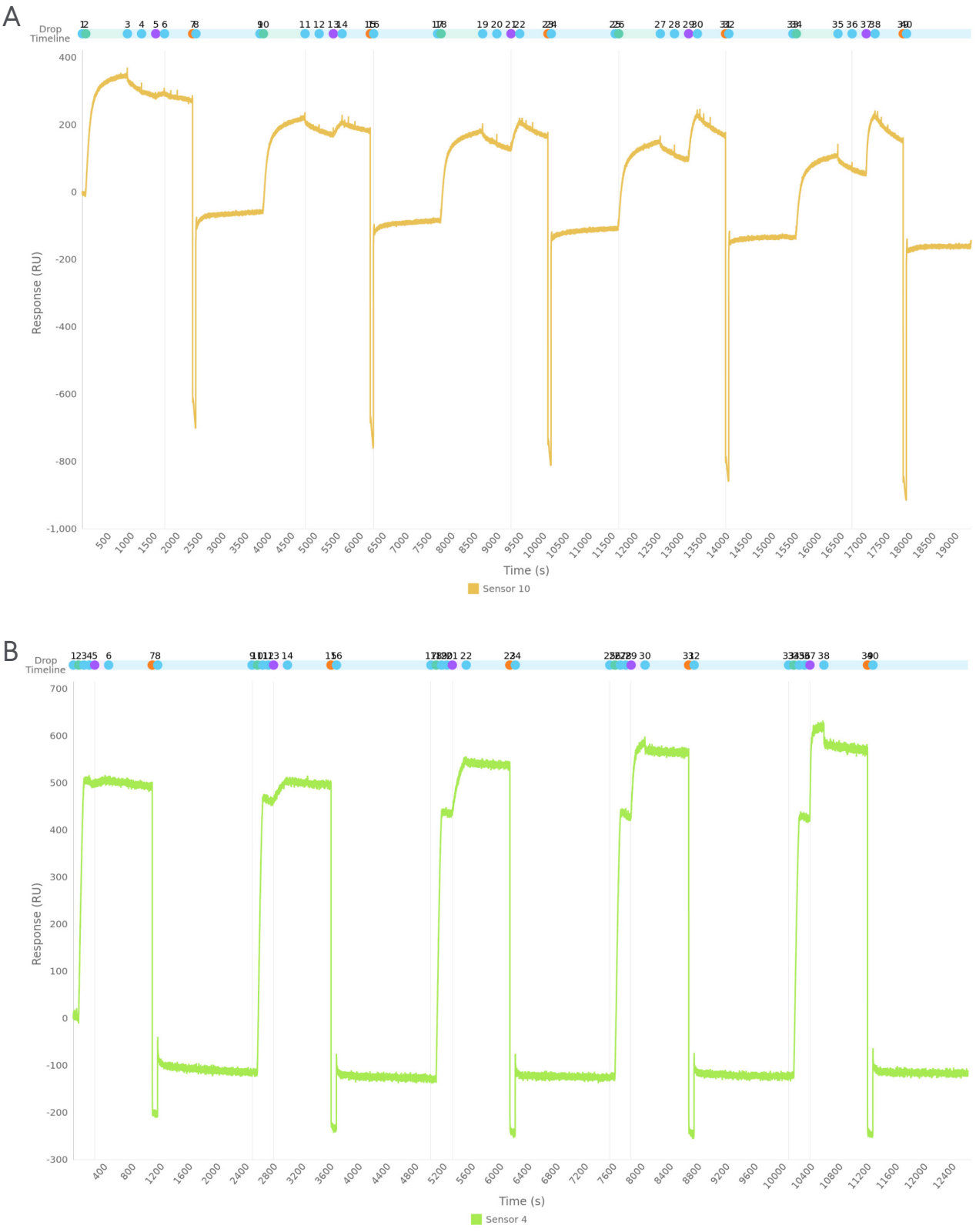

Figure 6: *Ligand loading and regeneration for the FcγR-IgG1 interaction. A) Anti-HIS antibody was immobilized on Nicoya Carboxyl Sensors followed by 5 cycles of FcγR binding kinetics. For each cycle 1 µg/mL of HIS-tagged FcγRI was captured on the anti-HIS surface followed by association and dissociation of the analyte, IgG1 Tocilizumab, and regeneration with 10 mM glycine-HCl, pH 1.5. Tocilizumab was analyzed at concentrations of 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM and 100 nM. B) Protein A was immobilized on Nicoya Carboxyl Sensors followed by 5 cycles of FcγR binding kinetics. For each cycle 5 µg/mL of Bevacizumab was captured on the Protein A surface followed by association and dissociation of the FcγRI analyte and regeneration with 10 mM glycine-HCl, pH 1.5. The FcγRI was analyzed at concentrations of 1.2 nM, 3.7 nM, 11.1 nM, 33.3 nM and 100 nM (a small bulk shift is detected for the highest concentration of the FcγRI analyte).*

Results & Discussion

In this study, Alto compared the binding kinetics of various FcγRs against two different IgG1 binding partners using anti-HIS, protein A, and biotin-streptavidin capture methods. The capture of the HIS-tagged FcγRs on the anti-HIS surface, Bevacizumab on the Protein A surface, and the bt-FcγRs on the streptavidin surface were optimized to obtain a low ligand density while allowing for detection of all analyte concentrations sampled. Figure 6A shows an example of multiple cycles of HIS-tagged FcγRs capture on the anti-HIS surface, with each immobilization followed by association and dissociation of Tocilizumab and regeneration of the sensor surface. Figure 6B shows an example of multiple cycles of Bevacizumab capture on the Protein A surface, followed by association and dissociation of the FcγR and regeneration. Full regeneration is achieved for both methods as well as consistent recapture of the ligands to the surfaces.

Kinetic values were calculated using a 1:1 Langmuir fit since FcγR binding to IgG has been shown to be a 1:1 interaction in previous studies.[7,8] However, for the lower affinity FcγRII and FcγRIII interactions complex binding profiles may be observed, such as biphasic dissociation phases, that may affect the 1:1 fit.[9] To reduce the complex binding profile, follow best practices, such as keeping the ligand density low or shortening the association time. Also to improve the fit of the data, the user can crop the dissociation phase to fit only the initial off rate from the biphasic curve (Figure 7).

The kinetics parameters calculated for the anti-HIS and protein A capture methods are shown in Tables 1. The calculated data are similar across both assay methods and agree with previously reported literature values for FcγR/IgG1 kinetics.[10] Looking closer at the data for the various FcγRIIA and FcγRIIIA variants, we can see that Alto was able to detect expected differences in kinetics. The FcγRIIA 167H variant showed a slightly stronger affinity for IgG1 compared to the 167R, likewise the FcγRIIIA 176V variant was found to have a higher affinity for IgG1 compared to the 176F, as previously reported in literature.[10] Figure 8A-C shows representative sensorgrams for IgG1 binding to HIS-tagged FcγRI, FcγRIIA, and FcγRIIIA.

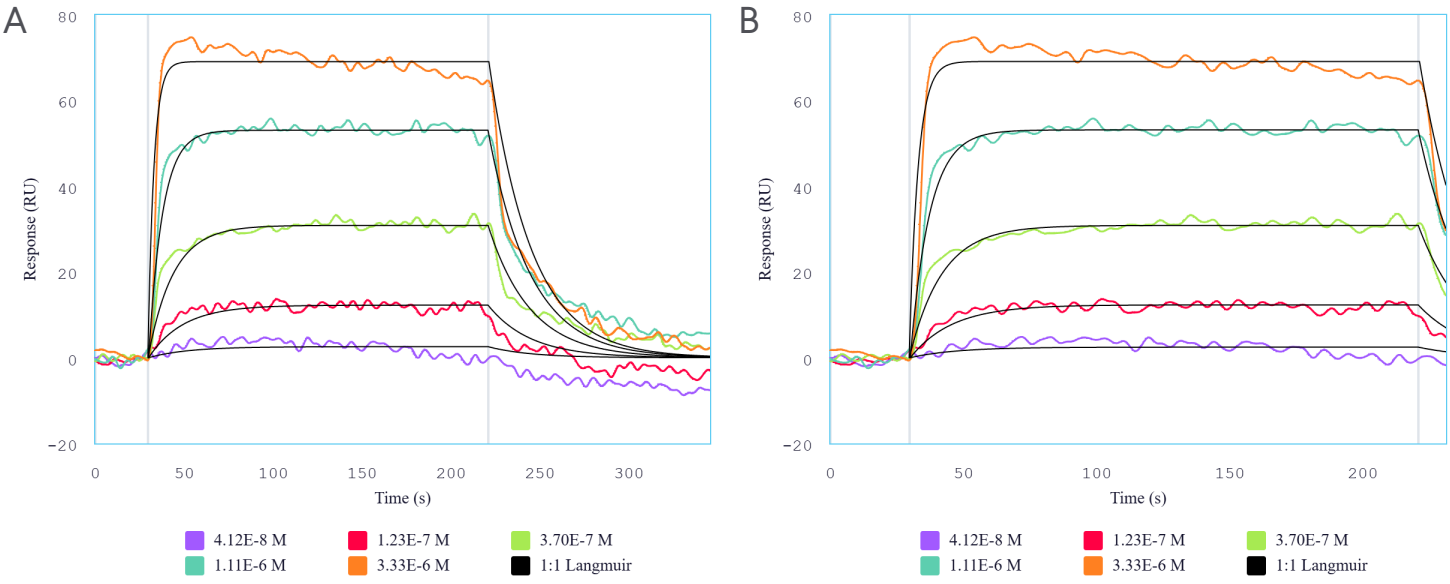

Figure 7: *Example fit of FcγRIII-IgG1 interaction using (A) full dissociation phase and (B) shortened dissociation phase. Using the start of the dissociation phase for fitting FcγRII or FcγRIII interactions with IgG shows improved fitting.*

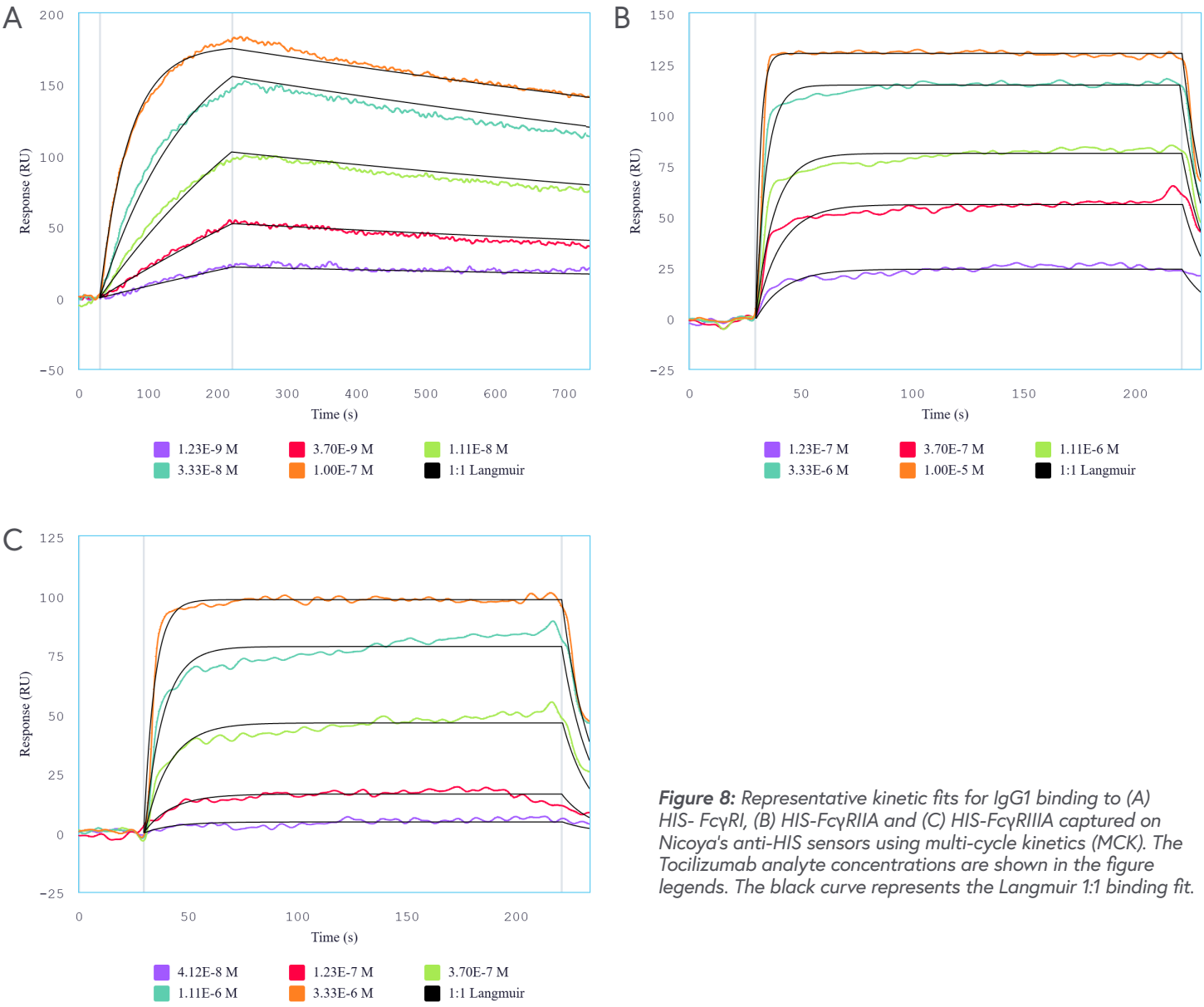

Figure 8: *Representative kinetic fits for IgG1 binding to (A) HIS- FcγRI, (B) HIS-FcγRIIA and (C) HIS-FcγRIIIA captured on Nicoya's anti-HIS sensors using multi-cycle kinetics (MCK). The Tocilizumab analyte concentrations are shown in the figure legends. The black curve represents the Langmuir 1:1 binding fit.*

Conclusions

SPR is a critical tool in the discovery and development of novel biological drugs. Alto eliminates the common challenges of using SPR to provide a more cost-effective and efficient solution for biologics discovery and development. Here, we have demonstrated approaches to measure high-quality kinetics of FcγR and IgG1 interactions using popular assay methods on the Alto. The DMF platform offers great flexibility in assay design and requires only 2 µL of sample for full kinetics. Effective assay design, optimization of the immobilization method, ligand density, analyte concentration and regeneration solutions are critical to running successful assays that give the most reliable data possible. By properly designing experiments and optimizing these critical parameters, kinetic analysis of FcγR-IgG interactions on the Alto Digital SPR system will consistently produce robust data and accurate, reliable kinetic parameters.

			Anti-His HIS-FcγR Capture			Protein A Bevacizumab Capture		
Molecule	Tags	FcR Type	k_a (1/M*s)	k_d (1/s)	K_D (M)	k_a (1/M*s)	k_d (1/s)	K_D
CD16a(176F)	His	FcγRIIIA	8.42×10^4	2.68×10^{-2}	324 nM	4.09×10^4	2.43×10^{-2}	1.32 µM
CD16a(176V)	His	FcγRIIIA	1.20×10^5	1.37×10^{-2}	114 nM	1.75×10^5	5.19×10^{-2}	300 nM
CD16b(NA1)	His	FcγRIIIB	4.71×10^4	6.16×10^{-2}	1.39 µM	3.13×10^4	1.61×10^{-2}	622 nM
CD16b(NA2)	His	FcγRIIIB	3.12×10^4	5.25×10^{-2}	1.91 µM	5.67×10^3	2.36×10^{-2}	4.61 µM
CD32a(167R)	His	FcγRIIA	7.19×10^4	6.70×10^{-2}	1.02 µM	4.97×10^4	2.88×10^{-2}	702 nM
CD32a(167H)	His	FcγRIIA	7.47×10^4	4.55×10^{-2}	667 nM	7.52×10^4	1.99×10^{-2}	232 nM
CD64	His	FcγRI	2.49×10^5	1.14×10^{-3}	4.94 nM	9.49×10^4	1.43×10^{-4}	1.52 nM

			bt-FcγR captured		
			k_a (1/M*s)	k_d (1/s)	K_D
CD16a(176F)	biotin	FcγRIIIA	8.81×10^3	1.24×10^{-2}	1.52 µM
CD16a(176V)	biotin	FcγRIIIA	9.18×10^3	1.02×10^{-2}	1.11 µM
CD16b(NA2)	biotin	FcγRIIIB	4.28×10^3	1.50×10^{-2}	3.49 µM

Table 1: *Summary of kinetic parameters for FcγRs measured on Alto*

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