Chapter 12

Kinetic Analysis and Epitope Binning Using Surface Plasmon Resonance

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Abstract

The ability to quantify binding affinity of molecular interactions is an essential component of drug development and life science research. This chapter outlines the practical use of surface plasmon resonance spectroscopy to monitor protein-protein interactions with an emphasis on basic experimental design. A short summary of epitope binning assays is also included.

Key words Surface plasmon resonance, Biosensor, Affinity, Binding kinetics, Ligand, Analyte, Epitope binning

1 Introduction

Surface plasmon resonance (SPR)-based biosensors allow the real time monitoring of interactions between proteins or other molecules without the use of labels. Antibody characterization is a typical application [1]. One interaction partner (referred to as the ligand) is immobilized on the surface of a sensor chip. The other interactant (the analyte) is passed over the surface in solution via an integrated microfluidic system. A light source is used to record a change in refractive index of the buffer near the sensor surface. Binding generates a response in resonance units (RU), which is proportional to the bound mass and plotted as a function of time in a sensorgram [2]. Association is observed when analyte flows over the surface and dissociation is monitored when buffer replaces the analyte flow. Regeneration of the ligand surface removes bound analyte at the end of each analysis cycle.

In most platforms incident light traverses a high refractive index medium (a prism) before it is reflected at a thin gold film on a glass surface without traveling through the sample on the opposite side of the film. This geometry is called a Kretschmann configuration [3]. Colored or turbid samples can be measured since

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no interference occurs from sample absorption or scattering, respectively. The sensorgram shape can be used to determine the association- (k_a; M^{-1} s^{-1}) and dissociation (k_d; s^{-1}) rate constants of the interaction. The ratio k_d/k_a defines the equilibrium dissociation constant K_D (M), which can be determined in the millimolar to picomolar range [4]. In contrast to equilibrium-based assays such as enzyme-linked immunosorbent assay (ELISA) detailed information about complex formation and stability can be collected. Several excellent reviews provide comprehensive guidelines on experimental design and interpretation of results [5–8].

The first part of this chapter describes the basic steps in experimental design and evaluation of a typical kinetic experiment. It comprises the following steps: immobilization of the ligand on the sensor chip surface, measurement of real-time interactions with the analyte, regeneration of the surface for subsequent injections and data evaluation. Commercially available systems provide sensor chips with different numbers of surface spots that can be used to immobilize ligand. Some chips are tailored for specific applications. Newer generations of array-based instruments expand the throughput of conventional SPR sensors and offer the ability to measure more reactions in a multiplexed format [9–11].

In the second part of the chapter a brief overview of SPR-based epitope binning strategies is given. Efficacy of antibodies is commonly attributed to their binding epitope and affinity. Unlike many other biophysical properties including affinity, the epitope is an inherent property that cannot be engineered [12, 13]. In epitope binning, binding molecules, typically antibodies, are assayed for their pair-wise ability to bind to an antigen. Simultaneous binding indicates that the pair recognizes two topologically distinct epitopes whereas interference indicates that binding occurs to the same or overlapping epitopes. Samples with the same blocking profile are grouped into bins. Antibodies that belong to the same epitope bin are likely to share similar functional characteristics. Thus, delineation of epitope specificities is an important component in focusing candidate selection on representative antibodies from each bin. Binning experiments are of a more qualitative nature compared to measurement of binding kinetics. Since the number of interactions grows very fast when larger panels of antibodies are studied, the assays are generally limited by throughput.

2 Materials

The procedures described in this chapter are general and should be implementable on several SPR platforms such as Biacore (3000, T200 etc.), Bio-Rad (ProteOn XPR36), SensiQ (Pioneer), or similar.

1. Sensor chip for amine coupling (e.g., Biacore CM5, Bio-Rad GLC, or equivalent).
2. Running buffer:
   (a) Phosphate-buffered saline (PBS) containing 10–50 mM sodium phosphate, 0.15 M NaCl, and 0.005% polysorbate 20 at pH 7.4.
   (b) HEPES (4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) buffered saline (HBS) containing 10–50 mM HEPES, 0.15 M NaCl, and 0.005% polysorbate 20 at pH 7.4.
3. Sample vials or plates with corresponding seals (instrument specific).
4. Preconditioning solutions (instrument specific).
5. Immobilization buffers:
   (a) 10 mM sodium acetate (NaOAc) buffer pH 4.0.
   (b) 10 mM NaOAc buffer pH 4.5.
   (c) 10 mM NaOAc buffer pH 5.0.
   (d) 10 mM NaOAc buffer pH 5.5.
   Dissolve 2.05 g (NaOAc 82.03 g/mol) in 50 mL deionized water for a 0.5 M stock solution. Adjust pH using acetic acid and dilute to 10 mM.
6. 0.4 M N-hydroxy-succinimide (NHS) or N-hydroxysulfosuccinimide (sulfo-NHS) in water (choice depends on instrument provider).
7. 0.1 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in water.
8. 1 M ethanolamine-HCl, pH 8.5.
9. Ligand solution in buffer free of primary amines.
10. Solution of capture agent in buffer free of primary amines (if a non-covalent immobilization approach is utilized).
11. Analyte solution (preferably in running buffer).
12. Regeneration solutions (interaction dependent).

3 Methods

Make sure the instrument is cleaned thoroughly before starting an experiment. Cleaning and routine instrument maintenance is the key to generating good quality SPR data. Filter all buffers and degas if the instrument does not have a built-in degasser. Reagent solutions should be freshly prepared and samples filtered. Recommended flow rates and contact times may differ between instruments, please refer to specific guidelines for each system.

Two recommended running buffers are HEPES buffered saline (HBS) and phosphate buffered saline (PBS) at pH 7.4. The buffers should include at least 100 mM salt to avoid nonspecific electrostatic interactions and a low concentration of detergent such as polysorbate 20 to prevent adsorption of analyte to vials and tubing.

3.1 Kinetic Analysis

3.1.1 Surface Preparation

This method uses carbodiimide/hydroxysuccinimide chemistry to activate carboxyl groups within the polymer coating on the sensor surface for covalent attachment of ligands via free amine groups [14] (*see* **Note 1**). This is the most generally applicable method for protein immobilization. The coupling procedure consists of three major steps: (1) activation, (2) coupling, and (3) deactivation (*see* Fig. 1). Sensor surfaces that do not require chemical activation such as streptavidin or metal chelating chips for capture of biotinylated or His-tagged proteins, respectively, are available from several suppliers. In cases where covalent attachment results in loss of ligand activity or is unsuitable for other reasons, immobilization of a capturing molecule provides an alternative approach (*see* **Note 2**). It is recommended that one channel be used as a reference for subtraction of responses due to bulk shift (change in refractive index between analyte solution and running buffer) or nonspecific binding. Surface preparation can typically be performed on all flow cells of the sensor chip simultaneously or only on one flow cell intended for use.

For kinetic measurements it is recommended to use the lowest amount of immobilized ligand that will give a measurable response. This reduces limitations on binding rates imposed by mass

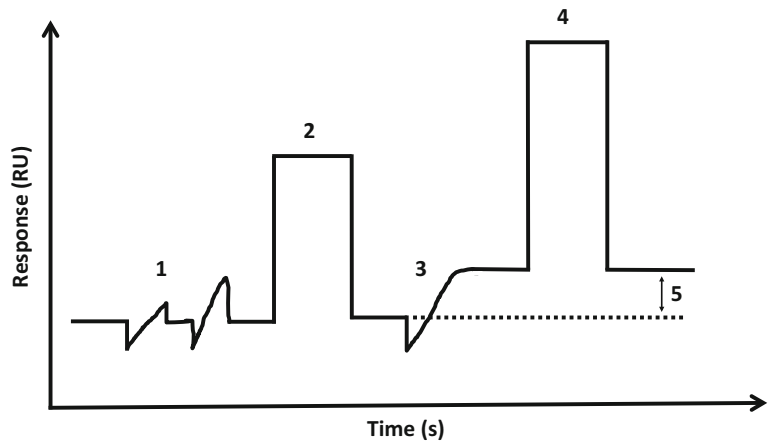

Fig. 1 Schematic sensorgram during surface preparation. Ligand pre-concentration is seen as a slope in response when ligand is injected over an unactivated surface (1). Once immobilization conditions (pH and ligand concentration) have been established the surface is activated (2), ligand is injected to a desired response level (3), and the remaining reactive groups are deactivated (4). The immobilization level is recorded from the sensorgram (5)

transport of the analyte to the surface and can improve the kinetic measurements. Deciding which interactant to immobilize and the appropriate immobilization level are important considerations, *see* **Note 3** for general recommendations. The activity of the ligand surface as well as the reproducibility of the response level should be assessed.

1. Prepare fresh running buffer at ambient temperature, take out an aliquot for sample dilution, and degas. Put the bottle in the buffer compartment and set the instrument temperature (usually 25 °C).
2. Insert and initialize a sensor chip that has previously been equilibrated to room temperature. Chips designed for low immobilization levels are generally used for kinetic analysis. Some instruments include a preconditioning step to be performed on newly docked sensor chips.
3. Prime the system two times with running buffer.
4. To determine the optimum conditions of pH and concentration for immobilization, ligand is injected over the unactivated sensor chip surface under different buffer conditions. Dilute each ligand to 10 μg/mL in 10 mM NaOAc pH 4–5.5, the pH should be above 3.5 (the pK_a of carboxyl groups on the surface) and at least 0.5–1 unit below the isoelectric point [15] of the ligand [15] in order for the surface and ligand to carry opposite net charges (*see* **Note 4**). Use 10 mM NaOAc pH 4.5 if pI is not known, this buffer works for many proteins. Inject a small volume (~30 μL) at a low flow rate (typically 10 μL/min) to test pre-concentration (*see* Fig. 1). Ligand solutions should be prepared in immobilization buffer as shortly as possible before use. The instrument control software typically calculates the minimum volume required for an injection by adding dead volumes.
5. Mix equal volumes of 0.4 M EDC and 0.1 M NHS and inject ~70 μL at a low flow rate to activate the spots on the senor chip surface intended for immobilization (*see* **Note 5**).
6. Prepare solutions at concentration and pH that yields efficient pre-concentration for each ligand. It is recommended to use a protein ligand with similar size and charge for the channel to be used as a reference surface.
7. Inject ligand to a desired immobilization level. Use a low flow rate and vary the contact time to reach the desired immobilization level. Many instruments offer automated immobilization protocols designed to immobilize ligand to a specified response level. Several subsequent injections can be made to reach the desired immobilization level (*see* **Note 6**).

8. Block excess reactive groups by injecting ~70 μL 1 M ethanolamine-HCl pH 8.5 at a low flow rate.
9. If one channel is immobilized at a time, repeat **steps 5–8** until all flow cells to be included in the experiment have been treated.
10. Stabilize the surfaces by injecting running buffer for 2 min at 100 μL/min. This step washes away non-covalently bound ligand. If a new ligand with unknown stability to common regeneration conditions is used, it is recommended to avoid using acidic or basic wash solutions until analyte binding has been tested (*see* **step 13**).
11. Note the immobilization levels (*see* Fig. 1). If a drifting baseline is observed, leave the system with standby flow of running buffer for 30–60 min.
12. Prepare an analyte dilution at a concentration where binding is expected (typically 10–1000 nM for antibody-antigen interactions). Dilute analyte in running buffer identical to the one in the buffer compartment to minimize bulk effects.
13. Test surface activity and response level by injecting analyte for 3 min at 50 μL/min. Start with a lower concentration and increase concentration if no or weak signal is observed. In the following steps, it is recommended to use analyte concentrations that yield maximum responses to more easily detect incomplete regeneration or loss of binding capacity.
14. Regenerate the surface by injecting a short pulse (~15–20 s) of regeneration solution at a high flow rate (100 μL/min). Regeneration is the process of removing bound analyte from the immobilized ligand. It is important to verify ligand activity before the surface is exposed to regeneration solution if regeneration affects ligand functionality. Check that the signal returns to baseline and that a new analyte injection yields similar response as in the previous step (*see* **Note 7**). Common regeneration solutions are dilute acids. 10 mM glycine pH 1.5–2.5 or 10–20 mM HCl often regenerate antibody-antigen interactions without damaging most ligands (*see* **Note 8**). Sometimes, several short pulses of regeneration solution are required and it is good practice to include a short stabilization period after regeneration. Regeneration may not be necessary for interactions with fast dissociation rates.
15. Repeat five analyte injections followed by regeneration to confirm a reproducible response and efficient regeneration. If several analyte/ligand pairs are included in the experiment this test should be performed for all combinations. Include buffer injections to enable double referencing, i.e., subtraction of signals from reference channel and buffer injection, when the surface performance tests are evaluated. If nonspecific binding is observed the buffer composition may be changed (*see* **Note 9**).

3.1.2 Analyte Injection

An analyte injection has four steps: (1) injection of running buffer to provide a baseline, (2) association phase when analyte is flowed over the surface, (3) dissociation phase when running buffer is flowed, and (4) regeneration to remove bound analyte. The most commonly used assay format involves multiple cycles where different concentrations of antigen or buffer are injected in series. An alternative strategy is to use a kinetic titration where analyte is injected from low to high concentration separated by short dissociation times. A longer dissociation phase is monitored after the last injection [16]. This approach and other specialized protocols such as injection of a full analyte dilution series in a "one-shot kinetics" experiment available on the ProteOn XPR36 [17] or "OneStep" analyte gradient injections on the SensiQ [18], are useful for interactions that are difficult to regenerate or when regeneration is detrimental to the ligand.

As discussed in the previous sections, optimal assay conditions to measure rate constants should be designed to minimize mass transport limitations. This is achieved by using low ligand densities (with R_{max} typically <100 RU) and high flow rates (50–100 μL/min). Excellent examples exist where high quality data have been collected at very low maximum responses [7, 19]. A well-known (active) analyte concentration is crucial since it directly affects the accuracy of the determined association rate constant and thereby the measured affinity (K_D).

The steps of a typical kinetic experiment are summarized below. Interaction of analyte with several ligands inmmobilized in different channels can usually be monitored in each injection.

1. Stabilization of a newly immobilized chip using a constant flow of running buffer overnight is recommended. This can help resolve initial drift problems.
2. Prime the system with buffer two to three times before starting analyte injections.
3. Prepare a series of analyte dilutions in running buffer (*see* **Note 10**). A general recommendation is to cover a range from 0.1–10 K_D and use two- or threefold serial dilution. The number of concentrations is typically 4–7 and concentrations are chosen to get an even spread of the curves. If possible the injection order should be randomized and replicate samples should always be included.
4. Inject analyte sample. Injections times between 2–5 min with a flow rate of 50–100 μL/min generally work well (*see* **Note 11**). Monitor dissociation for 5–30 min (*see* **Note 12**) depending on what was observed during regeneration scouting. Prepare several injections of running buffer using the same settings to enable double referencing.

5. Regenerate the surface(s) using previously established regeneration conditions.
6. Inject a short pulse of running buffer and let the chip stabilize for 1–2 min with a flow of running buffer to wash out the microfluidic system and allow the ligand to recover from regeneration.
7. Repeat steps 4–6 until all the samples have been injected. Injection of a list of samples can typically be automated in the instrument control software.

3.1.3 Data Evaluation

Interaction kinetics is obtained by fitting the binding data to a model that describes the interaction (*see* Fig. 2). Instruments usually come with dedicated analysis software packages with varying degrees of manual control, which make curve fitting straightforward. Generally, a simple Langmuir 1:1 model should be used to determine the kinetic constants. It assumes that the analyte is monovalent and homogeneous, that the ligand is homogeneous, and that all binding events are independent without mass transport limitation. Inability to fit data to a simple model is often a result of poor experimental design or heterogeneous interactants [7]. More complex models will invariably fit the data better but may hide potential artifacts that cause complex sensorgrams. Such models should only be used when other experiments can validate a more complex interaction scheme.

Prepare Sensorgrams

1. Overlay binding curves obtained at four to seven analyte concentrations.

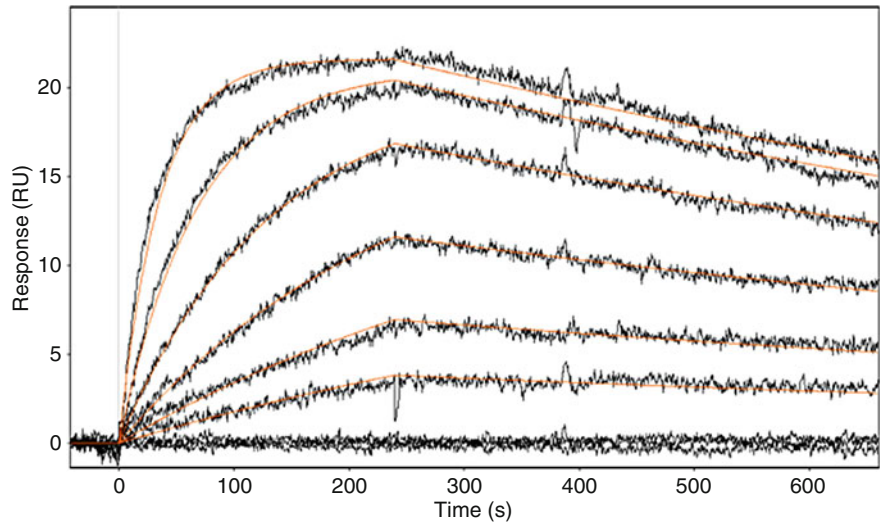

Fig. 2 Representative sensorgrams from an analyte dilution (black curves) with overlaid fitted curves calculated from a 1:1 model (red). Six analyte concentrations were injected and two buffer blanks

2. Align the baseline (y-axis) immediately before analyte injection to zero. Align injection start (x-axis) and remove artifacts (air spikes that often occur at the start and end of the association phase).
3. Subtract responses from the reference channel and blank buffer injection. Double referencing can correct for artifacts such as bulk refractive index changes, drift, and nonspecific binding. Bulk refractive index differences result in square-shaped responses (*see* **Note 13**). Nonspecific binding typically gives increasing responses that never reach steady state.
4. Use the procedure above to overlay replicate analyte injections to verify that assay performance has been reproducible throughout the experiment.

Apply Kinetic Model

1. Choose appropriate interaction model. For a monovalent interaction with a covalently immobilized ligand a global 1:1 Langmuir model that simultaneously fits k_a and k_d can typically be applied. The formation of the ligand-analyte complex follows pseudo-first-order kinetics because the constant flow maintains an excess of analyte (negligible consumption) whereas the ligand concentration is kept constant. For data sets in which many analyte concentrations reach equilibrium during injection, K_D-values can be obtained from a plot of equilibrium responses against analyte concentration (*see* **Note 14**).
2. Define injection start, injection stop and select association- and dissociation phases. Include as much as possible of the association- and dissociation phases in the analysis.
3. Run fitting procedure to generate theoretical binding curves based on the experimental data. Based on an iterative comparison between the observed and calculated curves the software returns parameter values for k_a, k_d, and $R_{\max}$ that best describe the experimental data. Global fitting is recommended since it combines all information contained in each binding curve and therefore improves the statistical power of the analysis [20]. For kinetic titration all injections are analyzed in one sensorgram with a special Eq. [16].

Interpret Results

1. Visually inspect the overlay plot of the calculated binding curves and the experimental curves. The fitted lines should pass through the experimental sensorgrams.
2. Curvature in the association phase is required for good fitting of k_a, particularly for higher analyte concentrations. A linear association phase is an indication of mass transport problems. Significant dissociation (at least 5% and monitored for a sufficiently long time) should be observed during the dissociation

phase for k_d to be assessed reliably. For dissociation rates below 10^{-4} this requires 10 min to several hours.

3. The residual plot (i.e., a plot of the difference between observed and calculated data over time) provides an alternative representation of the quality of the fit. For a good fit the residual plot should form a random scatter with the same magnitude as the noise level. Large deviations or trends (curvature) in the residual plot indicate uncertainty in the calculated parameter values. Smaller deviations are frequently observed in the association phase and should be viewed in relation to the experimental response levels.
4. Examine the parameter values: compare experimental R_{max} to calculated saturation response. If the R_{max} is very high compared to the response of the curves this can be an indication that the fit is wrong. Do values for k_a and k_d fall within a reasonable range (k_a 10^3–10^7, k_d 10^{-6}–10^{-1})? Is the affinity dissociation constant ($K_D = k_d/k_a$) realistic? Binding curves with the calculated characteristics can easily be simulated and compared to the experimental data to assess the credibility of the fit.
5. Curve fitting generally returns statistical parameters that describe the goodness of the fit. Chi^2 represents the average of the squared residuals. Empirically this value should be less than 5–10% of R_{max} [19]. Standard errors are commonly reported for the rate constants to describe how sensitive the fitting is to changes in these parameters. High SE-values for k_a may indicate mass transport limitation. The quality of a fit should be judged by several criteria (points 1–5 above) and not only be based on single statistical parameters.
6. From the initial 1:1 fitting one can consider using the baseline drift or mass transport model in order to improve the fit. Langmuir with drift is commonly used in experiments that use a capture surface where the capture ligand may dissociate from the capture agent on the chip surface. This can often be dealt with using an appropriate reference surface. Drift can also be caused by temperature fluctuations, which are more common in the beginning of an experiment [7]. Mass transport is a well-understood physical property of the system and partial mass transport limitations can be accounted for during data analysis. Some newer instruments incorporate mass transport in all models. However, testing for mass transport limitation by varying the flow rate is recommended [21].

In summary, experimental conditions should be optimized as far as possible to enable the use of a simple interaction model. Finding an optimum immobilization procedure for each system is

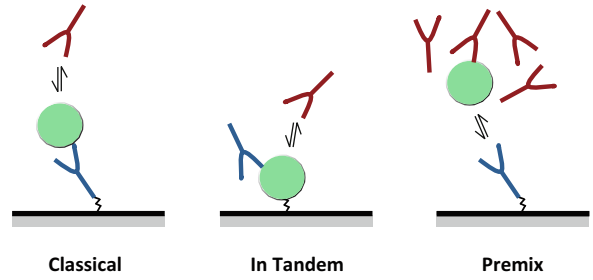

Fig. 3 SPR-based epitope binning strategies

usually the best way to do this. To this end different immobilization levels, immobilization chemistries, chip types, and analyte/ligand orientations may be evaluated. To improve quality, reliability, and reproducibility of reported biosensor data a list of minimum experimental conditions to be reported in publications has been proposed [1]. This includes (1) instrument used in analysis; (2) identity, source, and molecular weight of the ligand and analyte; (3) surface type; (4) immobilization condition; (5) ligand density; (6) experimental buffers; (7) experimental temperatures; (8) analyte concentrations; (9) regeneration conditions; (10) figure of binding responses with fit; (11) overlay of replicate analyses; (12) model used to fit the data; and (13) binding constants with standard errors or average parameter values from replicate measurements with standard deviations.

3.2 Epitope Binning

In principle there are three ways to do pair-wise epitope binning using SPR: classical sandwich binning, in tandem or premix [15] (Fig. 3). In the classical setup one antibody (Ab1) is immobilized followed by serial injection of the antigen and the second antibody (Ab2). Antibody immobilization may be covalent or by capture and the antigen has to be monomeric. For in tandem binning a saturating concentration of the first antibody is injected over immobilized antigen followed by an injection of competing antibody. Premixing involves incubation of antigen with an excess of one antibody followed by injection over the other antibody. Evaluation of binning results is not always straightforward and relies on the use of appropriate controls. In general, self-blocking should be demonstrated and at least two experimental setups should yield the same result. Binning can in principle be applied to investigate any competing protein-protein interactions. A common assay is to look at competition with natural protein ligands for their receptors. In contrast to epitope binning, epitope mapping requires a panel of custom-designed antigen mutants or peptides that cover the primary sequence or the antigen. Consequently, mapping experiments are much more demanding in terms of reagent requirements and throughput.

The basic steps in each binning strategy are summarized below. The assay formats are complementary but have different strengths and disadvantages.

3.2.1 Classical Sandwich Binning

1. Immobilize Ab1 using amine coupling (*see* Subheading 3.1.1) or by capture (*see* **Note 2**). Antibodies that dissociate rapidly from the antigen may be inappropriate candidates for coupling because there may be insufficient amounts of bound antigen to be recognized by Ab2. In general, weakly binding antibodies should be assigned the role of competing antibody (Ab2).
2. Inject a sufficient concentration of antigen to generate a binding signal. The antigen has to be monomeric in this assay and data interpretation is facilitated if response levels are high.
3. Inject Ab2. **Steps 2–3** can commonly be performed using a co-inject command, which fills the sample loop with two analytes separated by an air bubble. Buffer injections over the surface with Ab1 with antigen bound can be used to correct for antigen dissociation.
4. Subtract reference injections and evaluate the results. If the antibodies are directed toward distinct epitopes both should be able to bind simultaneously. Antibodies that bind interfering sites will compete with each other. This is detected as a lack of binding of Ab2.
5. To validate the results the assay should be repeated using the reversed order of Ab1 and Ab2. Self-blocking using the same antibody in both the steps should also be evaluated. If simultaneous binding is still observed it indicates that the antigen may be multivalent or, in the case of capture, that the capture agent is not fully saturated. A few issues may complicate the analysis (*see* **Note 15**).
6. Regenerate the surface before a new cycle is initiated.

3.2.2 In Tandem Binning

1. Immobilize the antigen using amine coupling (*see* Subheading 3.1.1). This strategy is compatible with multivalent antigens and in principle it also works for captured antigen if capture is made using a tag or a known non-interfering epitope. A risk with the in-tandem assay is that antigen immobilization may mask or interfere with epitopes.
2. Inject Ab1. This injection has to saturate all binding sites.
3. Inject Ab2. In cases when the dissociation rate of Ab1 is fast an excess of Ab1 can be included in the injection of Ab2.
4. Data analysis is based on whether binding of Ab2 can be detected. Significant increases in response indicate a non-overlapping epitope. Ability of Ab2 to bind immobilized antigen can be confirmed by replacing the injection of Ab1

with buffer and this may also be used for individual referencing for each Ab2. Reference subtraction using buffer in the second injection can be used to correct for dissociation of Ab1.

5. The order of Ab1 and Ab2 should be reversed and self-blocking confirmed to validate the results.
6. Regenerate the surface before a new cycle is initiated.

3.2.3 Premix

1. Immobilize Ab1 using amine coupling (*see* Subheading 3.1.1) or capture (*see* **Note 2**). In contrast to the classical setup this strategy is compatible with multivalent antigens. Low affinity of Ab1 may be limiting in this setup.
2. Premix antigen (usually at a concentration above the K_D for both antibodies in the pair) with a large excess of Ab2 (>10 times the K_D).
3. Following a sufficient time of incubation, inject the pre-formed complexes over Ab1. Inject only antigen as a control.
4. Significant decrease in binding compared to antigen alone indicates that Ab1 and Ab2 compete. Due to the increased mass of the pre-formed complexes relative to antigen alone, augmented signal implies simultaneous binding.
5. Test self-blocking and validate findings by reversing Ab 1 and Ab2. Failure to demonstrate self-blocking indicates that a higher excess of Ab2 may be required. If capture is used the injection of Ab1 has to be saturating (*see* **Note 14**).
6. Regenerate the surface before a new cycle is initiated.

Data from all possible pairs are collected and used to map which antibodies recognize which epitopes based on how they can form blocking patterns with other antibodies. Weak binders can often give ambiguous results when studied in some assay orientations, such cases may be identified by switching the roles of the two antibodies. When the number of antibodies is small, experiments with varying concentration of Ab2 aimed to see dose-dependent signal increase/decrease could be used for validation purposes.

Binning of large antibody panels is challenging since many interactions need to be assayed and several potentially conflicting pair-wise results need to be evaluated. To perform a rough binning of many antibodies using fewer ligand surfaces, all antibodies can be competed with one selected Ab1 in the classical assay. Ab1 should have a slow dissociation rate. All antibodies that are blocked by Ab1 are grouped into bin 1. From the remaining antibodies a new antibody (Ab2) is selected and immobilized. The competition assay is repeated with all the remaining antibodies (i.e., antibodies not blocked by and binned together with Ab1) versus Ab2 and blockers are binned together. This procedure is repeated until all antibodies have been assigned to a bin. The premix assay can be

utilized to confirm the results using the same ligand surfaces. Major blocking groups can be distinguished using this strategy. However, it may fail to identify similar yet unique epitopes since the discriminative power is lower compared with an assay where all possible antibody pairs are interrogated [22].

4 Notes

1. The immobilization buffer should not contain primary amines (e.g., Tris or sodium azide) since these will compete for activated succinimide esters on the sensor chip surface with the N-terminus and ε-amino groups on lysine residues on the ligand. Alternative immobilization strategies should be considered for acidic proteins with pI values <3.5.
2. Capturing allows the same surface to be used for analysis of interactions involving different ligands. Ligand capture is sometimes referred to as non-covalent immobilization or indirect immobilization. A capturing agent is covalently immobilized and used to capture fresh ligand in each cycle. Regeneration of the surface typically involves removal of analyte together with ligand. Several suppliers offer specialized kits for capture of antibodies or tagged proteins. The use of well-defined capture agents makes it easier to find suitable regeneration conditions. Since the capturing step also provides a small-scale purification, the ligand purity is less important compared to covalent immobilization. Capture is an attractive approach when many antibodies are to be screened or when purified samples are not available (for example when screening hybridoma supernatants). The capturing molecule is generally immobilized to a relatively high level (>5000 RU when antibodies are used) and low amounts of ligand are required. Captured ligands often exhibit a high binding activity as a result of the oriented, homogeneous surface presentation. The interaction between the ligand and capturing molecule should be sufficiently high to ensure that little ligand dissociates from the surface for the duration of an analysis cycle. Use of a reference surface with only the capture agent is recommended to detect bulk shifts and nonspecific binding. If antibody dissociates from the capture surface during an antigen injection, the signal drift can be subtracted by double referencing with a cycle consisting of ligand capture followed by buffer.
3. The binding capacity of the surface depends on the immobilization level and activity of the ligand. This in turn determines critical parameters such as maximal response, possible mass transfer issues (i.e., when diffusion from the bulk solution to the surface is limiting analyte binding) and stoichiometry of the

system. The theoretical saturation response R_{max} (in RU) can be calculated from the molecular weights of the analyte and the ligand, the amount of immobilized ligand (R_L), and the stoichiometry of binding (n; defined as the number of binding sites on the ligand) (*see* Eq. 1).

$$R_{max} = \frac{M_w\,(\text{analyte})}{M_w\,(\text{ligand})} \cdot R_L \cdot n \qquad (1)$$

The experimental maximum response will be lower because all ligand is not active. The protein may not be 100% pure and all binding sites are not accessible for analyte binding. By calculating the R_{max} one can determine whether the ratio of the molecular masses of the interactants would limit the response. The immobilization level should be low for kinetic analysis, generally an $R_{max} < 100$ RU is recommended. An appropriate ligand density to aim for (R_L) can be calculated by rearranging Eq. 1. Kinetic rate constants are inherent to the interaction and, provided that the experiments are well designed, should be the same regardless of immobilization level and which binding partner is immobilized. The interaction should be measured in both analyte/ligand orientations when an accurate determination of affinity is critical and the interactants display 1:1 binding.

If one binding partner has more than one binding site (e.g., antibodies, Fc-fusion proteins or GST-fusions) it should be immobilized to avoid avidity effects. If a multivalent analyte is injected over a monovalent ligand, each binding site of the analyte can interact independently with the ligand surface. This results in erroneously slow dissociation rates (low values for k_d) and overestimated affinities. When monovalent antibody fragments are used as analytes, note that some fragments may have a tendency to oligomerize in solution [23]. In such cases lower ligand densities or capture of antibody fragment followed by injection of monovalent antigen may be required.

A final consideration is the concentration and availability of the interactants. Analyte concentration has to be accurately known to calculate association rate constants and it must be available over a suitable concentration range. The sample consumption for ligand immobilization is lower and the measured kinetic data are independent of its concentration.

4. Increase ligand concentration or decrease pH if pre-concentration is not observed. Ligand solutions for immobilization are usually dilute (10–50 μg/mL) provided that efficient pre-concentration can be achieved. Electrostatic pre-concentration is favored by low ionic strength (10–20 mM monovalent cations) in the immobilization buffer.

For ligands with pI above 7, maleate buffer pH 6 can be used for immobilization.

5. Thaw activation chemicals on ice and use immediately. The degree of surface activation will affect the amount of ligand immobilized on the surface and can be controlled by the concentration of EDC/NHS (dilute 1:1 mixture of coupling chemicals in water if immobilization level is too high). The exposure time to the reagents may also be varied. Alternative covalent immobilization chemistries (e.g., thiol or aldehyde coupling) can be utilized by various modifications of the activated surface groups.
6. The coupling efficiency will decrease as consecutive pulses of ligand are injected. The coupling reaction should be completed within 15–20 min after activation.
7. Do not compare baseline responses before analyte injection with response levels immediately after regeneration. Regeneration solutions with extreme pH values may cause temporary swelling/contraction of the polymer matrix that can affect response levels [24]. The evaluation should focus on finding reproducible analyte binding signals.
8. Incomplete regeneration or loss of ligand activity will impair the performance of the assay. The regeneration procedure is the key to get good data, it has to be evaluated empirically since the combination of physical forces responsible for the binding is often unknown. Always start with mild regeneration conditions and proceed successively to harsher conditions. It is not uncommon that the response in the first analyte injection is somewhat higher than what is observed in the following cycles. For more details on regeneration conditions and strategies to evaluate regeneration efficiency, see Nikolovska-Coleska or Drake and Klakamp [8, 25]. The number of times a sensor surface can be regenerated is often greater than 100 and depends on the nature of the ligand.
9. If nonspecific binding to the reference surface is observed this may be minimized by the modification of the sample and running buffers. Electrostatic nonspecific binding can be reduced by increased concentration of salt (NaCl); hydrophobic nonspecific binding can be reduced by the addition of a detergent (i.e., <0.1% polysorbate 20 or a low concentration of CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) [8]. In the case of persistent nonspecific binding the analyte/ligand orientation may be reversed.
10. Analyte is typically serially diluted in running buffer. Bulk shift problems may occur if the analyte concentration is high and it has a buffer composition that differs from the running buffer. If referencing does not resolve significant bulk problems, the

sample should be buffer exchanged into running buffer. Samples that contain high refractive index components such as dimethyl sulfoxide (DMSO) or glycerol should be avoided or their concentration matched with the running buffer.

11. A well-behaved binding curve is an exponential that reaches a plateau when the association and dissociation rates are the same. When buffer replaces analyte flow, exponential dissociation of the complex should be observed. The association phase should display enough curvature to allow the fitting model to estimate k_a reliably. It is recommended that the analyte injection time allows at least one analyte concentration to reach steady state. Linear association curves also at higher concentrations indicate that the system is limited by mass transport. To test whether binding is limited by mass transport the kinetics should be measured using different flow rates (and if possible with different immobilization levels).
12. Significant dissociation must be observed for an accurate determination of the dissociation rate constant. As a rule of thumb, at least 5% signal decrease should occur before regeneration. If dissociation is very slow the "short and long" strategy can be applied where the highest tested concentration is used to obtain a long dissociation time while dissociation is monitored only for a shorter time for the other curves. Note that this strategy requires an equivalent buffer injection for referencing.
13. If data that have been properly reference-subtracted show square-shaped response shifts at the beginning and end of injection, try setting the bulk response parameter to a constant value of zero [6].
14. For binding curves that reach steady state during injection, the affinity can be obtained from a plot of the equilibrium response (R_{eq}) versus the analyte concentration (c) (*see* Eq. 2). It is important to only include curves that have reached equilibrium and select a region of the curve where the response is flat (i.e., when association rate equals dissociation rate). A wide range of concentrations should be included to obtain reliable K_D values (typically 0.1–10 K_D).

$$R_{eq} = \frac{c \cdot R_{max}}{c + K_D} \tag{2}$$

15. A prerequisite for both the classical sandwich setup and the premix assay is that no nonspecific binding occurs between Ab1 and Ab2. This can easily be tested in reference injections that omit antigen. Complete blocking of a capture surface is needed to prevent capturing of the second antibody. This can be difficult to achieve and may involve an injection of a high concentration of an unrelated antibody before or at the same

time as antigen is injected. The saturation level should be determined experimentally for Ab1 if a capture approach is used. Allosteric antibodies that alter the antigen conformation and thereby influence the epitope of the second antibody pose unique challenges.

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