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# Expanding the ProteOn XPR36 biosensor into a 36-ligand array expedites protein interaction analysis

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ABSTRACT

Here we demonstrate methods to expand the throughput of the ProteOn XPR36 biosensor allowing for the simultaneous kinetic characterization of several multiplexed formats, such as 36 disparate antibodies targeting the same antigen, and facilitating detailed epitope binning and mapping studies. The kinetic rate constants determined by these methods correlated with those obtained on Biacore 2000 and the absolute parameter values obtained on the ProteOn's alginate-based GLC chip agreed closer with those from Biacore's flat C1 chip than Biacore's dextran-based CM4 chip. Pairwise epitope binning data from the ProteOn 36-ligand array format and those generated on an orthogonal array-based biosensor, the Octet QK384, gave similar results. In an epitope mapping study using biotinylated peptides, all three biosensor platforms were similar in their ability to identify antibodies that bound to linear epitopes. We apply alternative formats of the ProteOn array that enable a significantly higher number of assays to be conducted simultaneously than previously anticipated on this platform.



Label-free real-time biosensors are commonly used to characterize the interactions between diverse biomolecules and are cited in more than 1400 peer-viewed publications annually [1]. Most biosensors employ surface plasmon resonance (SPR)[1] in the classic Kretschmann configuration [2] to detect the binding of a solution partner (analyte) to an immobilized partner (ligand) on a solid support. Traditional Biacore platforms, such as 2000, 3000, and T100 (and T200, the recent update to the T100), employ a "one-on-many" approach by delivering one analyte at a time over three serially addressed ligand-coated surfaces and a reference surface. Array-based SPR-imaging biosensors employ a one-on-many approach with up to hundreds of immobilized ligands, such as those commercialized by Biacore (Flexchip), Genoptics, Plexera, and IBIS. Emerging platforms are now routinely multiplexed in that they deliver multiple analytes over multiple ligands simultaneously, enabling "many-on-many" approaches, in an effort to meet the increasing demands of drug discovery for higher throughput [3]. For example, Biacore A100 (and Biacore 4000, the recent update to the A100) [4] analyzes 16 interactions at once via the use of four independent flow cells that each contain four ligand spots and a reference spot. ForteBio's Octet QK384 and Red384 are based on biolayer interferometry and each processes 16 interactions simultaneously on independent disposable fiber-optic sensor tips that dip into and read samples arrayed within the wells of a microplate, thereby eliminating the need for the microfluidic delivery of samples [5]. Each of these multiplexed technologies enables higher-throughput, higher-data-content assays than those previously possible.

This study describes applications of another multiplexed technology, namely BioRad's ProteOn XPR36 platform, which addresses interactions on 36 reaction spots simultaneously by combining the use of six parallel flow channels with a microfluidic pathway that can rotate between vertical and horizontal directions [6]. This creates a two-dimensional interaction array within which the intersecting and nonintersecting flow paths define the reaction and reference spots, respectively. We demonstrate how to expand the number and diversity of interactions that can be addressed on the ProteOn by treating the surface as a 36-ligand array to exploit its full ligand capacity. For example, the array can contain 36 unique ligands, or fewer ligands immobilized under various conditions. We compare the throughput that can be achieved when this platform is used in a one-on-many format, with similar assays performed on orthogonal platforms, namely the Biacore 2000 and Octet QK384. Our examples draw from three applications that are important in the drug discovery of monoclonal antibodies: kinetic characterization, epitope binning, and epitope mapping.

In 2006, Karlsson et al. described an algorithm for fitting the binding responses of an analyte concentration series that is delivered in order of increasing concentration over the same ligand surface without regenerating it between successive analyte injections

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[1] *Abbreviations used*: BSA, bovine serum albumin; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; mAbs, monoclonal antibodies; MES, 2-(*N*-morpholino)ethanesulfonic acid; SNHS, sulfo-*N*-hydroxysuccinimide; SPR, surface plasmon resonance.

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and referred to this methodology as "kinetic titration." They described its use in the context of one-on-many biosensors (Biacore 2000, 3000, S51, and T100 instruments) and concluded that, "kinetic titration has the potential to improve throughput and may be particularly suitable for the parallel analysis required by protein arrays" [7].

In the same year, Bravman et al. described a "one-shot" method for analyzing kinetic data on the ProteOn biosensor, which involved immobilizing six ligand strips along whole channels in the vertical flow paths and then delivering the members of an analyte concentration series along parallel channels in the horizontal flow paths [6]. While this generates analyte-binding data on 36 reaction spots simultaneously, it yields full kinetic profiles of only six analyte/ligand pairs because the information from six replicate reaction spots along a whole channel is combined to construct a full kinetic profile of each analyte/ligand interaction. This is a fast way of analyzing the kinetics of one analyte binding to six immobilized ligands without the need to regenerate the surfaces (Fig. 1A), but multiple sensor chips and/or multiple runs are required when there are more than six unique interactions in the test panel.

Here we combine a 36-ligand array with the kinetic titration method to study 36 unique analyte/ligand pairs simultaneously without any regeneration, by injecting the members of an analyte concentration series in succession from low to high concentration through the same flow channel, so that an entire analyte concentration series interrogates each spot. The kinetic titration method thus enables the use of various multiplexed assay formats. For example, six different analytes can be flowed perpendicularly across six ligand strips, giving a six-on-six analyte-on-ligand format (Fig. 1B). The scope of the kinetic titration method can be altered to a one-on-36 analyte-on-ligand format by arraying ligands onto individual reactions spots instead of immobilizing them along whole channels (Fig. 1C). To further diversify the amount of information that can be obtained per chip, ligands that target the same analyte can be arrayed onto the spots that lie within the same channel to enable six unrelated panels of one-on-six to be studied simultaneously (Fig. 1D); this format can also be used to study other combinations, such as three unrelated panels of one-on-12 or two unrelated panels of one-on-18.

## Materials and methods

All experiments were performed at 25 °C using HBST as the running buffer [10 mM Hepes, pH 7.4, 150 mM NaCl, and 0.05% (v/v) Tween 20] unless noted otherwise. A ProteOn XPR36 biosensor equipped with GLC and GLM sensor chips and coupling reagents (10 mM sodium acetate, pH 4.5, sulfo-*N*-hydroxysuccinimide [SNHS], 1-ethyl-3-(3-dimethylaminpropyl)-carbodi-

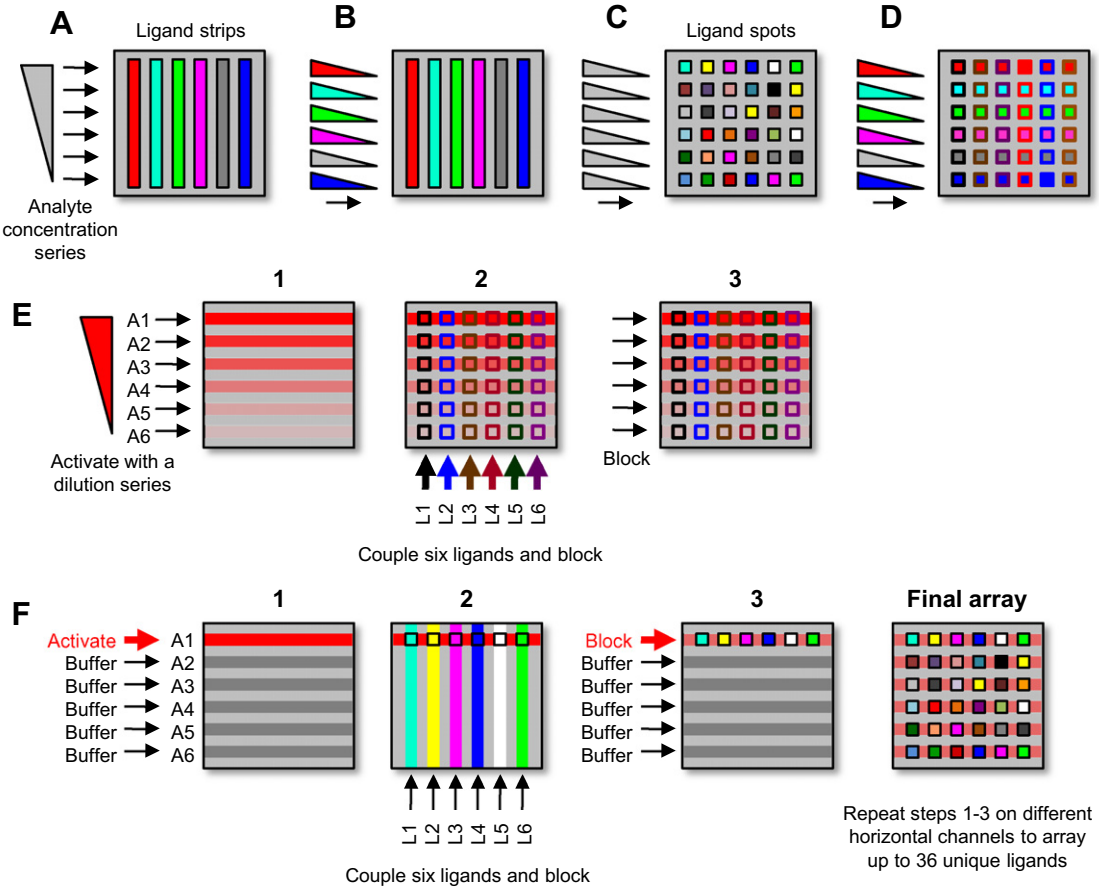


**Fig.1.** Single spot analysis diversifies the "analyte-on-ligand" kinetic assay formats that are available on the ProteOn. (A) "One-shot" kinetics gives a "one-on-six" format. Kinetic titration enables (B) a "six-on-six" format when six analytes (distinguished by the different colored triangles) are flowed over six ligand strips, (C) a "one-on-36" format when ligands are arrayed onto individual spots, and (D) six unrelated panels of "one-on-six" when six analytes are flowed along horizontal channels where their respective ligands are arrayed. Arraying (E) six ligands each at six capacities or (F) up to 36 unique ligands. Methods E and F involve rotating the flow path two and 12 times, respectively; fewer rotations are needed if less than 36 unique ligands are arrayed. In the ProteOn Manager control software, the six horizontal and six vertical channels are referred to as Analyte (A1–A6) and Ligand (L1–L6) channels, respectively. The reaction spots within the array can therefore be identified by their Analyte/Ligand coordinates, e.g., reaction spot A1L1 is located at the intersection of channels A1 and L1. Both methods create two types of interspot surfaces for local referencing, namely "activated and blocked" along the horizontal flow paths (represented by the orange strips) and "unmodified chip" along the vertical flow paths.

imide hydrochloride [EDC], and ethanolamine) was purchased from BioRad Inc. (Hercules, CA). An Octet QK384 biosensor equipped with amine-reactive, anti-murine-IgG-Fv, and streptavidin tips was purchased from ForteBio Inc. (Menlo Park, CA). A Biacore 2000 biosensor equipped with CM5, CM4, and C1 research-grade sensor chips, amine-coupling reagents (10 mM sodium acetate, pH 5.0, NHS, EDC, and 1 M ethanolamine HCl, pH 8.5), Biacore Mouse Antibody Capture Kit (containing rabbit polyclonal anti-mouse IgG that cross-reacts to rat IgG and 10 mM glycine, pH 1.7, regeneration buffer), and Biotin CAPture kit (containing CAP chips, Biotin CAPture solution, and regeneration reagents) were purchased from GE Healthcare (Piscataway, NJ). UltraPure Tween 20 and 2-(*N*-morpholino)ethanesulfonic acid [MES] were purchased from Sigma. ImmunoPure IgG elution buffer (pH 2.8) and streptavidin were purchased from Pierce Co. (Rockford, IL). Murine monoclonal antibodies were generated in-house via hybridoma technology. Kinetic studies were performed on a 13-kDa monomeric antigen (Ag1) that was prepared in-house and binning was performed on a 60-kDa monomeric antigen (Ag2) that was purchased from R&D systems (Minneapolis, MN). A "biotide" library consisting of 24 peptides each 15 amino acids long, overlapping one another by 10 residues, and modified at the N-terminal with a biotin linker and at the C-terminal with an additional glycine amide, was custom-ordered from JPT Peptide Technologies GmbH (Berlin, Germany); lyophilized biotides (about 60 nmol each) were reconstituted to 1 mg/ml in 100% DMSO prior to use. Free biotin was purchased from Vector Laboratories, Inc. (Burlingame, CA).

*Creating ligand arrays on the ProteOn for kinetics*

Immobilizations were performed at 30 μl/min on a GLC chip. In order to scout for appropriate immobilization levels for multiple ligands, six full-length IgGs were each coupled at six levels on a single sensor chip (Fig. 1E). The activation reagents (at stock concentrations of 0.4 M EDC and 0.1 M SNHS in water) were each diluted 50-fold in water and mixed together to give final concentrations of 8 mM EDC in 2 mM SNHS which provided the top concentration of a six-membered 2-fold serial dilution series with the most dilute sample representing a 1600-fold dilution of the stock solutions (final 0.25 mM EDC in 0.063 mM SNHS). Separate horizontal channels (referred to as "Analyte" channels in the ProteOn control software) were activated for 3 min with different members of this dilution series to create an activation "gradient". Next, six IgGs were each diluted to 20 μg/ml in 10 mM acetate, pH 4.5, and coupled for 3 min along separate vertical channels (referred to as "Ligand" channels in the ProteOn control software) followed by a 3-min injection of ethanolamine to block the reaction spots. Another 3-min pulse of ethanolamine was injected in the horizontal direction to block the previously activated spots. Final immobilization levels ranged from 139 to 3628 RU on individual reaction spots. This method involved rotating the flow path twice and created two types of interspots, namely "activated and blocked" along the horizontal channels and "unmodified chip" along the vertical channels.

Twenty-nine unique IgGs were arrayed at low capacities on a GLC chip by diluting each of the activation stock solutions 600-fold in water to give a mixture containing 0.667 mM EDC in 0.167 mM SNHS, and injecting it immediately along a single horizontal channel for 3 min; at the same time, buffer was injected along each of the other five channels. Six IgGs were each diluted to 15 μg/ml in 10 mM acetate, pH 4.5, and coupled for 3 min along separate vertical channels. Ethanolamine was injected for 3 min along the vertical flow paths and then again along the horizontal flow paths to block the previously activated spots. The activation, coupling, and blocking steps described above were repeated on different channels, until the chip was coupled with a different IgG per reaction spot (Fig. 1F). Since we had fewer than 36 IgGs in our test panel, we coupled six of them onto duplicate reaction spots by activating two horizontal channels simultaneously, instead of one. One reaction spot in the array was left blank by injecting the 10 mM acetate, pH 4.5, buffer (without IgG) during the coupling step. This method resulted in IgG immobilization levels of 541 to 1310 RU on individual reaction spots and involved 10 rotations of the flow path. To array 36 unique IgGs on individual spots, 12 rotations would be performed.

*Kinetic titration on the ProteOn*

Samples of a 13-kDa monomeric antigen (Ag1) were prepared at final concentrations of 3.7, 11, 33, 100, and 300 nM and injected at 30 μl/min in order of increasing concentration along each horizontal channel (Fig. 1C). Association was monitored for 3 min, allowing a 30-s dissociation time for all samples except the top concentration, which was allowed to dissociate for 20 min. The actual time between successive injections was about 6 min because of the additional time needed for the instrument to prepare an "Analyte"-quality injection, as defined by the default parameters in the instrument control software. Surfaces were regenerated by applying two 18-s injections of a 2/1 (v/v) mixture of Pierce IgG elution buffer/4 M NaCl ("Pierce/salt cocktail") at 100 μl/min. The titration data were collected in duplicate binding cycles and preceded by a series of buffer injections that provided blanks for "double-referencing" purposes [8].

*One-shot kinetics on the ProteOn*

The immobilizations and kinetics were performed at 30 μl/min. Full-length IgGs were coupled at low levels onto separate whole vertical channels of a GLC chip by simultaneously activating all channels for 3 min with a freshly prepared mixture of EDC and SNHS, each at 1/600 or 1/800 dilution of their stock concentrations, and then injecting 20 μg/ml IgGs in 10 mM acetate, pH 4.5, for 3 min. Channels were blocked using a 3-min injection of ethanolamine. Final immobilization levels varied from 165 to 589 RU with <3% variation along a channel. A dilution series of Ag1 was prepared as above and its members were injected simultaneously along the horizontal channels for 3 min (Fig. 1A); buffer was injected along the sixth channel to provide an "in-line" blank for double-referencing purposes. Dissociation was monitored for 15 min and surfaces were regenerated as above. A typical experiment collected one-shot data in triplicate and experiments were repeated on independent chips.

*Kinetic titration on the Biacore via anti-mouse-captured IgGs*

A rabbit polyclonal anti-mouse IgG was immobilized on all four flow cells of a CM5 chip using standard amine-coupling chemistry at a flow rate of 5 μl/min. This involved activating the surfaces with a freshly prepared mixture of 200 mM EDC in 50 mM NHS for 7 min, coupling the polyclonal antibody at 30 μg/ml in 10 mM acetate, pH 5.0, for 7 min, and blocking the excess reactive esters with ethanolamine for 7 min. Final immobilization levels ranged from 12,400 to 14,000 RU. Twenty-nine mouse IgGs were each diluted to 2 μg/ml in running buffer and captured for 1 min at 10 μl/min onto separate flow cells, leaving one surface bare (just the capture molecule) to provide a reference channel; in this way, three IgGs were analyzed per binding cycle. The flow path was then directed over all surfaces and the flow rate set to 30 μl/min. Samples of Ag1 were prepared at concentrations of 3.7, 11, 33, 100, and 300 nM and injected in succession for 3 min each, allowing a 20-s dissociation time for all samples except the top concentration, which was allowed to dissociate for 20 min. The actual time interval between

successive injections was about 3 min due to the additional time needed for the instrument to prepare a KINJECT-quality injection. The capture surface was regenerated with a 3-min injection of 10 mM glycine, pH 1.7. Each IgG was captured in duplicate binding cycles, with buffer as the analyte in one cycle and Ag1 in the other, to provide a customized buffer blank per IgG for double-referencing purposes. Some IgGs were captured in three separate cycles to study analyte binding in two cycles and buffer in a third cycle and thus provide a duplicate kinetic measurement of each of these mAbs.

*Kinetic titration on the Biacore via amine-coupled IgGs*

IgGs were immobilized at low levels onto separate flow cells of CM4 or C1 chips via standard amine-coupling chemistry at 5 μl/min. A typical coupling cycle involved activating a flow cell for 7 min with a freshly prepared mixture of 200 mM EDC in 50 mM NHS, injecting 15 μg/ml IgG in acetate, pH 5.0, until about 800 RU (CM4) or 500 RU (C1) were coupled, and blocking excess reactive esters with a 7-min injection of ethanolamine. One flow cell per chip was left unmodified (CM4) or activated and blocked (C1) to provide a reference surface. Final immobilization levels ranged from 672 to 760 RU (CM4) and 191 to 509 RU (C1). Ag1 was injected as a five-membered 3-fold or 5-fold dilution series with a top concentration of 300 nM for 3 min at 30 μl/min using a kinetic titration mode as described above or a classical injection mode, in which every analyte sample was allowed to dissociate for 20 min and surfaces were regenerated after every analyte injection with two 30-s injections of the Pierce/salt cocktail. All samples were injected in duplicate binding cycles and experiments were repeated three independent times.

*Classic sandwich binning on the ProteOn*

Thirty-five IgGs (and a blank) were arrayed at high capacities onto individual reaction spots of a GLM chip as described above (in "Creating ligand arrays on the ProteOn for kinetics"), but using a more potent activation mixture, namely a 1/20 dilution of each activation reagent, which resulted in final immobilization levels of 3590–8330 RU. A "Coinject Analyte" command was used to deliver 60 nM 60-kDa monomeric antigen (Ag2) followed immediately by 100 nM IgG (the "sandwiching" IgG), along the horizontal flow paths, allowing each sample a 3-min association time. After a 3-min dissociation time, the surfaces were regenerated with two 18-s injections of the Pierce/salt cocktail at 100 μl/min. A full 36 × 36 binning matrix was accomplished by repeating the Ag capture and sandwiching steps, varying the sandwiching IgG per binding cycle to address a solution counterpart of every coupled IgG and thus incorporate a full set of "self-sandwich" controls where the solution and coupled IgG were the same. In some cycles, buffer was used in the sandwich step instead of an IgG to provide a "blank" that monitored the Ag's dissociation from each coupled IgG.

*Classic sandwich binning on the Octet*

Octet data were collected using an agitation speed of 1000 rpm. Sixteen IgGs were coupled onto individual amine-reactive sensors by activating the sensors for 5 min with a 1/40 dilution of the EDC and SNHS stock solutions (final mixture contained 10 mM EDC and 2.5 mM SNHS in 0.1 M MES, pH 5.0), coupling IgGs at 30 μg/ml in 0.1 M MES, pH 5.0, for 10 min, and blocking excess reactive esters with 1 M ethanolamine for 5 min. A new baseline was established in PBS + 0.05% (v/v) Tween 20 + 1 mg/ml BSA running buffer for 15 min. A full 16 × 16 binning matrix was accomplished in 18 binding cycles where each cycle involved immersing the IgG-coupled sensors in the following solutions: 100 nM IgG (to monitor nonspecific IgG/IgG cross-reactions), 100 nM Ag2 (to capture Ag), 100 nM IgG (the same one as earlier, to monitor sandwiching), and 15 mM phosphoric acid (to regenerate the sensors). The IgG and Ag were each allowed to bind for 250 s and the regeneration was performed by exposing the tips to three alternating rounds of 15 mM phosphoric acid (30 s) and buffer (20 s). The IgG/IgG cross-reaction step also served to monitor the efficiency of the regeneration step. Each of the coupled IgGs was used as the sandwiching IgG, thereby defining a full set of self-sandwich controls. Buffer was used to replace IgG in duplicate cycles to monitor the Ag's dissociation from each of the coupled IgGs.

*Epitope mapping on the ProteOn*

Twenty-four biotides were arrayed onto individual spots of a GLC chip using a modified version of the method that was used to directly couple up to 36 IgGs (see above). Thus, streptavidin was amine-coupled along a single horizontal channel by activating it with a freshly prepared mixture of EDC and SNHS, each at 1/20 dilution of their stock concentrations, injecting 50 μg/ml streptavidin in 10 mM acetate, pH 4.5, and blocking with ethanolamine; each of these injections was allowed a 3-min contact time. The flow path was rotated to the vertical direction and six biotides were captured at 1 μg/ml for 5 min along separate vertical channels followed by a 3-min injection of 1 μM biotin to block these reaction spots; another injection of biotide with no further capture confirmed that these reaction spots had been fully blocked. A 3-min injection of 1 μM biotin was then directed over the horizontal channel that had been previously coupled with streptavidin to block the horizontal interspots. The method was repeated on other horizontal channels, one by one, until all the biotides had been arrayed. Since we had fewer than 36 biotides, we arrayed six of them on duplicate reaction spots by coupling streptavidin onto two horizontal channels simultaneously instead of one and left one horizontal channel unmodified. The vertical interspots resulting from this method were "unmodified chip" and the horizontal interspots were "biotin-blocked streptavidin." Having arrayed the biotide library, 15 μg/ml IgG was injected along all six horizontal channels at 30 μl/min, allowing 2-min association and dissociation times. The surfaces were regenerated with two 18-s injections of 12 mM NaOH in 1 M NaCl at 100 μl/min. Sixteen different IgGs were tested in succession in this manner, duplicating one of them at the start and end of the assay to confirm that the assay was reproducible on multiple regenerations.

*Epitope mapping on the Octet*

Fifteen biotides were each diluted to 1 μg/ml in running buffer (HBST + 1 mg/ml BSA) and captured onto individual streptavidin tips for 10 min. At the same time, an anti-murine-Fv-specific tip was used as the 16th sensor and dipped into buffer. A new baseline was established for 10 min in running buffer and then all 16 tips were dipped into 15 μg/ml IgG for 5 min, allowing a 3-min dissociation time. Regeneration was accomplished via two 30-s exposures to 12 mM NaOH in 1 M NaCl (biotide/SA tips) or 75 mM phosphoric acid (anti-murine-Fv tip). Eight IgGs were analyzed in succession in this manner; one of them was tested in triplicate binding cycles to monitor the cycle-to-cycle reproducibility. The anti-murine-Fv tip served as a positive control to verify the presence of the solution-based IgGs, even for those that did not map to any biotide.

*Epitope mapping on the Biacore*

The Biotin CAPture kit was used as directed by the manufacturer. Fifteen biotides were each diluted to 2 μg/ml in HBST

running buffer. A typical binding cycle involved capturing the Biotin CAPture reagent (streptavidin conjugate) at 50 μg/ml onto all four flow cells for 5 min at 2 μl/min, capturing three biotides onto separate flow cells using a 1-min injection at 10 μl/min for each biotide, leaving the conjugate unmodified on one flow cell to provide a reference surface, and then injecting 30 μg/ml IgG for 2 min over all channels. After a 1-min dissociation phase, the Biotin CAPture reagent and captured biotide were removed by regenerating with a 3/1 (v/v) cocktail of 8 M guanidine-HCl and 1 M NaOH. Thirty-five binding cycles were run, varying the biotides and IgGs per run, thereby screening each of six IgGs against 15 biotides and analyzing one of the IgGs in duplicate over the entire set of 15 biotides.

### *Data analysis*

One-shot kinetics on the ProteOn were analyzed in ProteOn Manager v. 2.1. Processing of the reaction spot data involved applying an interspot-reference and a double-reference step using an in-line buffer blank. The processed data from replicate one-shot injections were fit globally to a simple Langmuir binding model without mass transport. Kinetic titration data collected on the ProteOn were processed in ProteOn Manager by concatenating the responses of a five-membered analyte (or buffer blank) dilution series, establishing a baseline response prior to the first injection and interspot-referencing the data. These processed data were then imported into BiaEvaluation v. 4.1 for further analysis, which involved double-referencing the data (by subtracting the buffer blank responses from the analyte-binding responses on a per spot basis) and fitting a kinetic titration model that includes a term for mass transport. Binning and mapping data collected on the ProteOn were interspot-referenced in ProteOn Manager. All Biacore data were analyzed in BiaEvaluation v. 4.1. Kinetic data were double-referenced by subtracting the responses from the reference surface ("across-channel" referencing) and the buffer blanks ("within-channel" referencing) and fitting these data globally to a simple binding model that included a mass transport term. For interactions that exhibited square-shaped binding responses, and thus lacked sufficient curvature to enable an accurate kinetic analysis, plots of the equilibrium binding response as a function of the analyte concentration were constructed and fit to a simple steady-state binding model. Mapping data obtained on the Biacore were processed by reference-surface subtraction; no double-referencing was needed because of the high binding responses and very stable peptide captures. Octet data were exported as text files and processed in BiaEvaluation, which involved Y-averaging the data to zero at the start of the binding step of interest without any reference subtraction.

## Results

### *Using the ProteOn as a 36-ligand array in kinetic studies*

Low capacity surfaces are recommended for generating high quality kinetic data on a biosensor [8]. Therefore, the ability to scout multiple ligand capacities for multiple interactions simultaneously streamlines assay development (Fig. 1E). Rotating the ProteOn's flow path between the activation and the coupling steps of an amine-coupling immobilization opens up the possibility to array six ligands each at six capacities in a rapid manner that takes 15 min longer than a conventional immobilization of six ligands along whole channels. This extra time is needed because the flow path is rotated twice in the former method while not at all in the latter with each rotation taking approximately 5 min; there is also an additional blocking step. In this way, we coupled six IgGs each at six capacities and delivered an analyte (a 13-kDa monomeric antigen, Ag1) over them in a kinetic titration mode (Fig. 1C), which allowed us to scout optimum capacities for determining the kinetics of these interactions. In general, only the spots coupled with less than 500 RU IgG yielded analyte-binding responses that conformed to a simple model, as judged by their low and randomly scattered residuals that had an amplitude comparable to instrument noise (approximately ± 4 RU). Thus, we were able to extract accurate kinetic rate constants for six disparate analyte/ligand pairs simultaneously without any regeneration. Fig. 2A and C show the overlay plots of the experimental and fitted data for two kinetically different IgGs that were each coupled onto individual spots; data from the two lowest capacity spots are shown per plot. To confirm that the kinetic titration method over discrete spots was reliable, we studied the same interactions via the conventional one-shot kinetic method over six whole channels, each coupled with a different IgG (Fig. 2B and D). The rate constants obtained from kinetic titration and one-shot methods were very similar to one another, as published elsewhere [7]. These data demonstrate that the array-based method can integrate an assay optimization step into the kinetic assay itself and thus explore 6-fold more ligand capacities than the one-shot method.

We extended the scope of the kinetic titration method to the simultaneous study of one analyte interacting with a larger panel of IgGs by arraying a different IgG onto each spot (Fig. 1F). Since we had fewer than 36 different IgGs in our test panel, we arrayed six of them in duplicate to monitor spot-to-spot variability within the array and intentionally left one spot blank (just activated and blocked) to provide a negative control surface to monitor any non-specific analyte binding or spot-to-spot leakage on coupling; we did not observe any binding to this reaction spot throughout the assay, as desired. We kinetically titrated Ag1 in the horizontal direction (the format shown in Fig. 1C), which accessed the activated and blocked interspots for locally referencing the reaction spot data. Directing the analyte along the vertical channels would have accessed the unmodified interspots; we found that both types of interspots were equivalent in this assay because the analyte did not interact with either of them, as desired. Arraying the ligands took approximately 3 h, injecting the buffer blanks took an hour, and injecting the analyte took an additional hour. Thus, in a total assay time of 5 h and without any regeneration we obtained full double-referenced kinetic profiles from 36 reactions spots simultaneously. Conveniently, all immobilized IgGs regenerated using the same conditions, allowing us to duplicate the analyte kinetic titration (in an additional hour) and thus demonstrate that the assay was highly reproducible on every spot. Fig. 3 shows examples of the high quality and kinetically diverse data obtained on individual spots, each coupled with a different IgG. Most of the response data were amenable to a kinetic analysis and some were also amenable to an equilibrium analysis because steady-state responses were achieved rapidly within the association phase of these mAbs (e.g., IgGs 15 and 20, for which only the affinities and not the kinetic rate constants are reported in Table 1).

We compared the throughput in obtaining kinetic parameters for the above-noted reagents using a capture assay on the Biacore 2000. All mAbs within the test panel were full-length mouse IgGs, so we captured them via a preimmobilized rabbit polyclonal anti-mouse IgG. The unattended run time of the assay was about 29 h based on a 67-min cycle time with an additional hour or two of hands-on user time that included preparing the instrument, immobilizing the capture reagent, and dispensing the samples.

Fig. 4 and Table 1 compare the kinetic parameters obtained for the same set of interactions studied by ProteOn and Biacore 2000, namely Ag1 binding a panel of 24 immobilized IgGs. While the kinetic rate constants determined by both technologies were highly correlated, as indicated by linear fits giving $R^2$ values close to 1

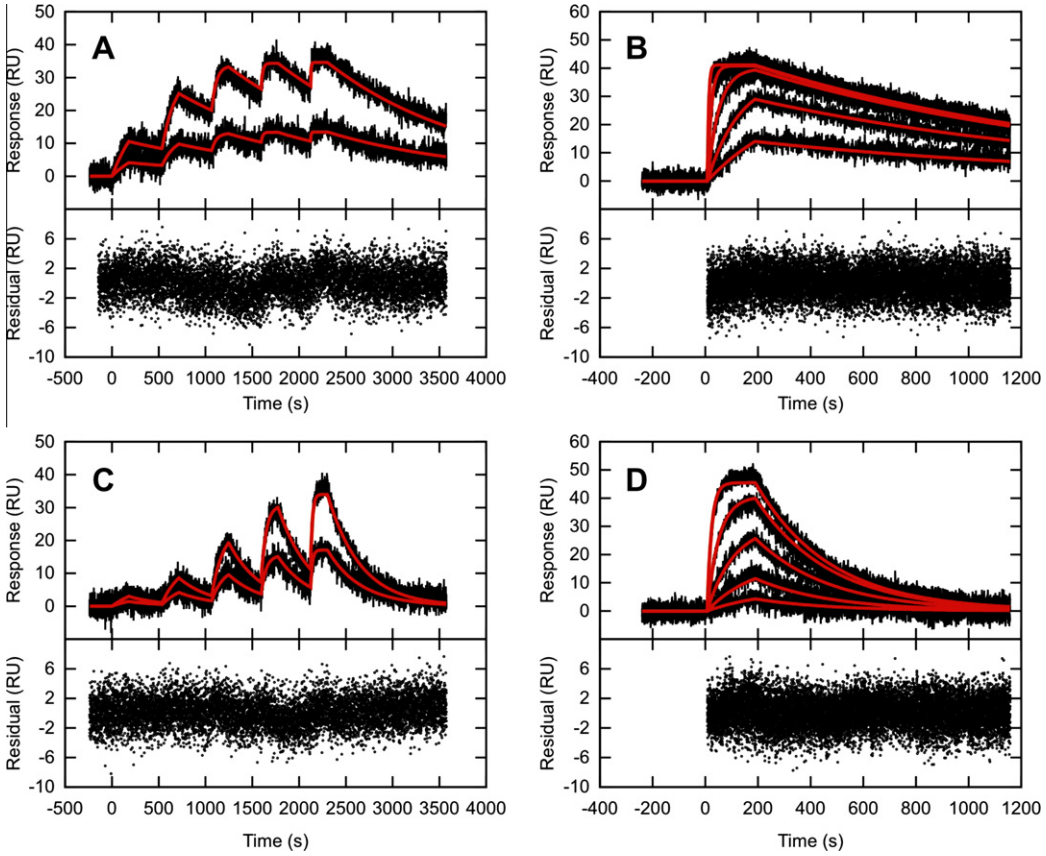


**Fig.2.** Comparing kinetic titration and one-shot kinetics on the ProteOn. Kinetic titration over mAbs arrayed onto individual spots at immobilization levels of (A) 139 and 285 RU mAb 1 and (C) 260 and 463 RU mAb 5. One-shot kinetics on whole channels coupled with mean levels of (B) 350 RU mAb 1 and (D) 590 RU mAb 5. The black lines represent the measured data and the red lines represent the global fits to a simple model with mass transport; the residuals are shown below each overlay plot. In each case, the analyte (Ag1) was injected as a five-membered 3-fold dilution series with a top concentration of 300 nM. The interactions shown in panels A–D were determined to have association rate constants ($\times 10^{-5}$ $M^{-1}$ $s^{-1}$) of 5.87 (0.07), 8 (1), 2.0 (0.4), and 2.1(0.3); dissociation rate constants ($\times 10^{3}$ $s^{-1}$) of 0.641 (0.009), 0.73 (0.02), 3.1 (0.4), and 3.4 (0.2); and affinities (nM) of 1.09 (0.02), 1.0 (0.2), 16 (4), and 16 (2). The kinetic titration and one-shot measurements represent the mean (and standard deviation) of two or four independent experiments, respectively.

(0.80 for $k_a$ and 0.87 for $k_d$), the association rate constants (Fig. 4A) were systematically higher on the ProteOn, as manifested by a slope significantly different from 1 (0.28), while the dissociation rate constants (Fig. 4B) were very similar, as manifested by a slope close to 1 (0.80). Therefore, the $K_D$ values (Fig. 4C) were systematically smaller on the ProteOn by a factor of 1.4 (as indicated by the slope of the linear fit in Fig. 4C). The black dotted line in Fig. 4C correlates the affinities for 14 mAbs that were analyzed kinetically, while the red dotted line correlates a larger data set that incorporated 10 additional mAbs whose affinities were determined via a steady-state analysis.

To determine whether the discrepancy in the $k_a$ was due to the use of different immobilization chemistries in the two assays, we studied Ag1's binding to three kinetically diverse mAbs (1, 7, and 16) by directly amine-coupling them onto a Biacore CM4 chip. Our results confirmed that the kinetics obtained on the Biacore were very similar whether we amine-coupled the IgGs onto a CM4 chip or captured them via preimmobilized anti-murine IgGs on a CM5 chip. To investigate whether the different surface chemistries employed on each platform caused the observed differences in the kinetic parameters (ProteOn's GLC chip has an alginate-based surface matrix, while Biacore's CM4 and CM5 chips have a carboxymethyldextran-based surface matrix), we studied these interactions on Biacore's flat dextran-free C1 chip. Panels A, B, and C of Fig. 5 compare the kinetics of Ag1 binding to mAb 16 that was amine-coupled onto three different chip types, namely Biacore CM4, ProteOn GLC, and Biacore C1, respectively. The kinetics and affinity plot shown in Fig. 5D reveals that the $k_a$ obtained for Ag1 interacting with three different mAbs (distinguished by symbol shapes) on the GLC chip (red symbols) more closely resembled those of Biacore's flat C1 chip (green symbols) than the dextran-based CM4 chip (black symbols); the $k_a$ agreed within 1.4-fold (comparing C1 to GLC chips) and 3.2-fold (comparing GLC to CM4 chips). For all three mAbs tested, the differences in the measured $k_a$ values across all three chip types were statistically significant (all $P$ values <0.05, Welch's $T$ test). For two of the three mAbs (7 and 16) the differences in the $k_d$ values were not significant ($P > 0.2$); for one mAb (1), the difference in the $k_d$ values was significant when comparing values from CM4 to C1 chips, or GLC to CM4 chips ($P < 0.003$). However, the absolute $k_d$ values across chip types varied less than the absolute $k_a$ values as visualized in Fig. 5D and summarized in Table 2.

### *Using the ProteOn as a 36-ligand array in epitope binning*

Characterizing a complete reactivity pattern matrix for pairwise epitope binning is challenging due to the geometric scaling of the number of interactions with the number of mAbs in the test panel [9]. For example, binning 10 mAbs requires 100 interactions to exhaust every pairwise permutation and binning 20 mAbs requires 400 interactions. By expanding the ProteOn into a 36-ligand array we were able to test the ability of 36 mAbs to form sandwich pairs with one another in a single assay that addressed 1296 interactions. The reagents employed for epitope binning were unrelated

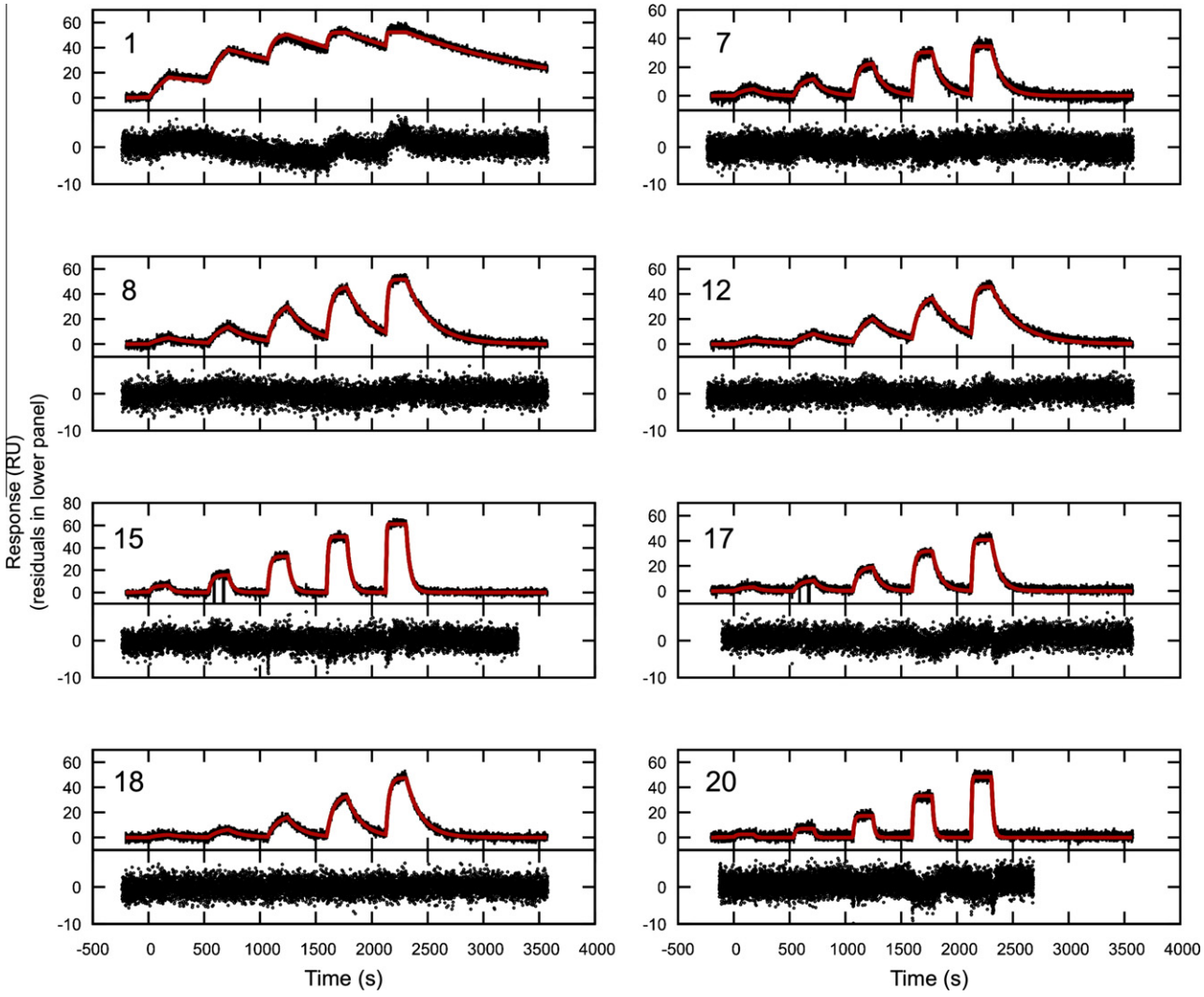


**Fig.3.** Examples of single spot kinetic analysis on the ProteOn. Each panel represents a global analysis of duplicate kinetic titrations of Ag1 at concentrations of 3.7, 11, 33, 100, and 300 nM over an individual spot coupled with a mAb (numbered as in Table 1). MAbs 7 and 20 were each coupled onto duplicate spots, so four curves are overlaid in those panels. The black lines represent the measured data and the red lines represent the global fits to a simple model with mass transport; the residuals are shown below each overlay plot. See Table 1 for the kinetic rate constants. Only the affinity and not the kinetic values are reported for mAbs 15 and 20 because their kinetics were too fast to measure accurately.

to those used in the kinetic study described above. We used a "classical sandwich" binning method because the Ag under investigation was a monomer; this method is restricted to monovalent Ags to ensure that the coupled and solution mAbs bind Ag simultaneously only if the two mAbs in question bind nonoverlapping epitopes [10]. Fig. 6A shows an overlay plot of 18 rounds of binning on a single spot coupled with one of the mAbs in the panel; similar plots were constructed for all other spots, which were each coupled with a different mAb. Interactions were classified as "blocked", "sandwiched", or "ambiguous" by visual inspection of these overlay plots. While a more quantitative method could have been employed via a statistical analysis of replicates, our simple method facilitated a rapid evaluation of each pairwise interaction that was performed in singlet.

The Ag capture was fairly reproducible on the majority of the coupled mAbs, as shown in Fig. 6A, confirming that they tolerated multiple rounds of acid regeneration, which was a key requirement of this assay. Some of the coupled mAbs appeared to lose activity on regeneration, but not enough to hinder their formation of obvious sandwich complexes. One of the higher affinity mAbs (23) was unaffected by the regeneration condition and the Ag remained bound to it throughout the entire assay. However, this did not adversely affect its ability to sandwich with other mAbs because the regeneration step completely removed the sandwiching mAbs from this complex since regeneration worked (at least partially) for all other mAbs in the set. On the whole, sandwich responses were easily discernable above the self-sandwich and buffer blank controls, with most sandwiching interactions giving responses at least 250-fold above the instrument noise (±4 RU). Based on a 15-min cycle time that involved coinjecting Ag and a sandwiching mAb and then regenerating the surfaces, it took 9 h to explore the full $36 \times 36$ binning matrix with an additional 3 h to array the ligands.

We performed a similar classical sandwich binning assay on the Octet QK384 to illustrate the throughput that could be achieved on this many-on-many platform. Since this instrument processes 16 interactions simultaneously, we selected 16 mAbs at random from the above panel and coupled then onto individual sensor tips. In addition to the "self-sandwich" and "buffer blank" controls that we had incorporated in our ProteOn assay, we included an IgG/IgG cross-reaction step that confirmed that none of the IgGs bound one another nonspecifically. This step also aided in identifying tips

**Table 1**
Kinetic analysis of Ag1 binding 24 immobilized mAbs that were amine-coupled onto a ProteOn GLC chip or captured via pre-immobilized polyclonal anti-murine IgG on a Biacore CM5 chip. The Biacore data were collected in two independent experiments while the ProteOn data were collected in a single experiment, except for six mAbs (1, 5, 7, 8, 15, and 16) that were each analyzed in two independent experiments; data from duplicate runs are separated by a comma. MAbs 11, 13, 14, 15, and 19–24 bound with square-shaped binding responses so their kinetics could not be defined precisely; the ratio of the kinetic rate constants ($K_D = k_d/k_a$) gave an affinity that was similar to that determined via a steady-state analysis of the similar binding responses.

| MAb No. | ProteOn | | | Biacore | | |
|---|---|---|---|---|---|---|
| | $10^{-5} \times k_a$ ($M^{-1}$ $s^{-1}$) | $10^3 \times k_d$ ($s^{-1}$) | $K_D$ (nM) | $10^{-5} \times k_a$ ($M^{-1}$ $s^{-1}$) | $10^3 \times k_d$ ($s^{-1}$) | $K_D$ (nM) |
| 1 | 5.92, 5.82 | 0.63, 0.65 | 1.1, 1.1 | 1.27, 1.69 | 0.52, 0.68 | 4.1, 4.0 |
| 2 | 10.80 | 4.22 | 4 | 3.00, 3.69 | 2.31, 2.38 | 8, 6 |
| 3 | 5.42 | 3.16 | 6 | 1.89, 1.66 | 1.60, 1.95 | 8, 12 |
| 4 | 4.63 | 3.84 | 8 | 1.73, 1.48 | 2.05, 2.20 | 12, 15 |
| 5 | 2.25, 1.67 | 3.45, 2.83 | 15, 17 | 0.80, 0.90 | 2.68, 2.57 | 33, 28 |
| 6 | 3.09 | 4.98 | 16 | 1.11, 1.20 | 3.22, 3.18 | 29, 27 |
| 7 | 5.44, 4.50 | 11.6, 13.1 | 21, 29 | 1.48, 1.92 | 9.14, 9.15 | 62, 48 |
| 8 | 2.12, 2.14 | 4.53, 4.35 | 21, 20 | 0.87, 0.95 | 3.58, 3.08 | 41, 32 |
| 9 | 5.83 | 12.9 | 22 | 2.78, 2.91 | 10.1, 6.96 | 36, 24 |
| 10 | 4.67 | 11.3 | 24 | 2.38, 2.20 | 12.2, 11.2 | 51, 51 |
| 11 | – | – | 29 | – | – | 91, 66 |
| 12 | 1.27 | 4.23 | 33 | 1.02, 0.62 | 4.89, 3.86 | 48, 62 |
| 13 | – | – | 35 | – | – | 54, 83 |
| 14 | – | – | 37 | – | – | 67, 61 |
| 15 | – | – | 38, 37 | – | – | 89, 80 |
| 16 | 1.07, 0.90 | 4.34, 4.05 | 41, 45 | 0.88, 0.63 | 4.14, 3.34 | 47, 53 |
| 17 | 2.80 | 13.9 | 50 | 1.07, 1.30 | 8.81, 12.4 | 82, 95 |
| 18 | 1.03 | 7.47 | 73 | 0.81, 0.51 | 7.25, 5.83 | 90, 115 |
| 19 | – | – | 80 | – | – | 186, 195 |
| 20 | – | – | 88 | – | – | 147, 124 |
| 21 | – | – | 116 | – | – | 206, 257 |
| 22 | – | – | 168 | – | – | 217, 283 |
| 23 | – | – | 170 | – | – | 328, 288 |
| 24 | – | – | 184 | – | – | 247, 266 |

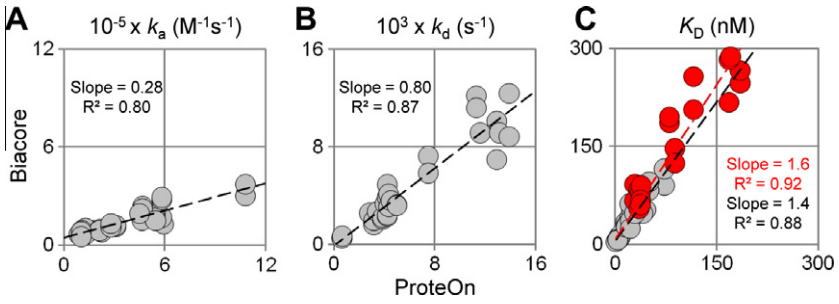


**Fig.4.** Correlation of kinetic parameters determined using ProteOn and Biacore technologies in a kinetic titration mode. (A) Association rate constants, (B) dissociation rate constants, and (C) equilibrium dissociation constants determined for Ag1 interacting with a panel of IgGs. Fourteen clones (gray circles) were analyzed kinetically and 10 clones (red clones) were analyzed via a steady-state analysis due to them lacking sufficient curvature in their binding responses to justify an accurate kinetic analysis. The ProteOn data were collected in a single array-based experiment (except six mAbs that were analyzed in two independent experiments) whereas the Biacore data were collected in two independent experiments. The slopes and correlation coefficients ($R^2$) of the dotted linear trend lines are shown on the plots. In panel C, the black line correlates the kinetically derived affinities for 14 mAbs whereas the red line correlates the affinities of 24 mAbs that include 10 additional mAbs whose affinities were determined via a steady-state analysis of their equilibrium binding responses. Only 24 of the 29 mAbs studied are shown here because the remaining five were determined by both technologies to have affinities too weak to determine precisely by the analyte concentration range used in the assay. See Table 1 for the kinetic rate constants.

that did not regenerate properly. For example, a tip coupled with mAb 23 did not regenerate at all after its first exposure to Ag, which meant that the Ag/IgG complex persisted throughout the entire assay, consistent with the behavior of this mAb in our ProteOn assay. Thus, the IgG/IgG cross-reaction step was instead interpreted as the binning step for this coupled mAb. A full $16 \times 16$ binning matrix was performed in an unattended run time of 6 h, which included the couplings that were integrated into the automated run; an additional hour of hands-on time was needed to prepare the samples. A $32 \times 32$ binning matrix would have taken four times as long and would have likely been unfeasible to run unattended due to issues related to evaporation, since the samples are accommodated in open microplates. Omitting the IgG/IgG cross-reaction steps would have shortened the run time and thus enabled a larger number of interactions to be explored unattended. Most of the coupled mAbs tolerated multiple rounds of acid regeneration as demonstrated in Fig. 6B, which shows an example of the primary data obtained on a single tip; 15 other tips each coupled with an unique mAb were processed simultaneously.

The final binning matrix from a single ProteOn assay is shown in Fig. 7A. Several bins were discerned (A–M listed across and down the matrix) based on their sandwiching profiles. The matrix was perfectly symmetric, confirming that the order in which the mAbs bound the Ag did not affect their ability to pairwise bind, as expected for a "well-behaved" homogeneous and monovalent Ag that does not undergo an allosteric change on binding of mAb. While data were collected for an entire 36-ligand array, only 31 mAbs were binned in Fig. 7A because we excluded data from three spots coupled with irrelevant mAbs (31, 32, and 35) that served as negative controls, a blank spot (just activated and blocked), and a spot coupled with mAb 2 because this mAb performed poorly in the assay as judged by its unusually high (and therefore unacceptable)

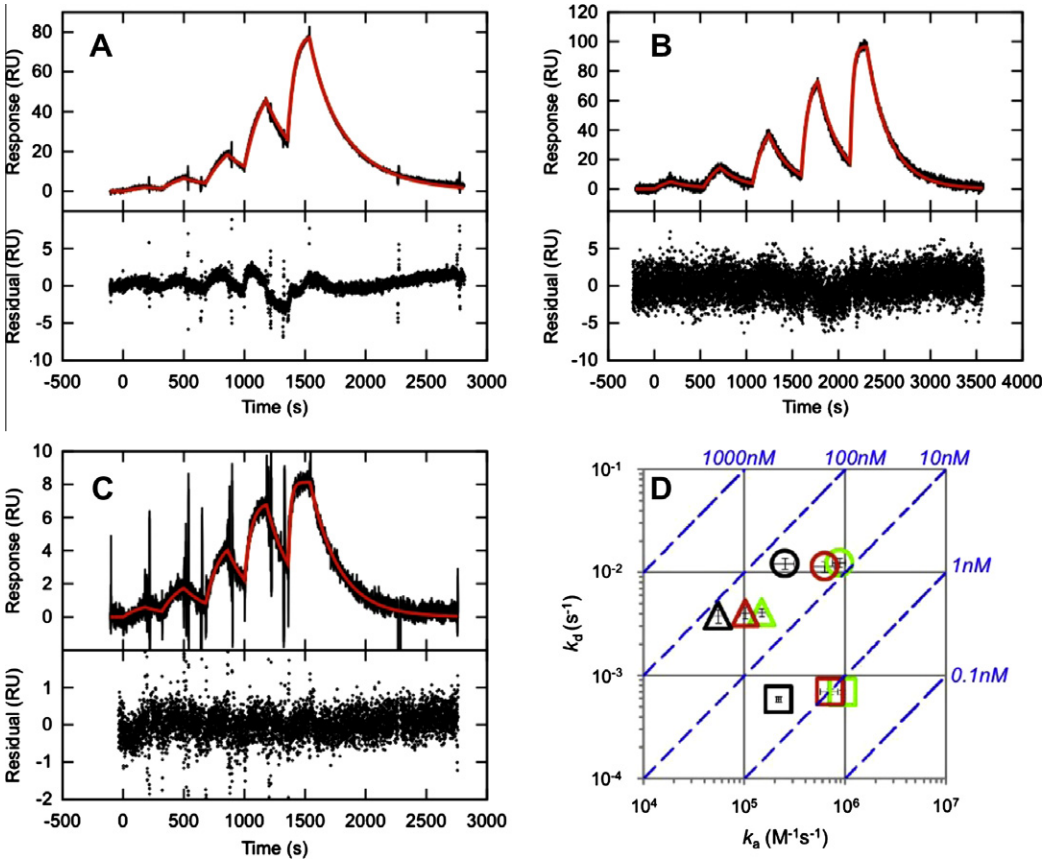


**Fig.5.** Binding kinetics of Ag1 (3.7, 11, 33, 100, and 300 nM) to mAb 16 amine-coupled onto (A) Biacore-CM4, (B) ProteOn GLC, and (C) Biacore-C1 chips. Each of these panels show a global analysis (red lines) overlaid with the primary data (black lines) for duplicate kinetic titrations. (D) Kinetics and affinity plot for three kinetically diverse mAbs (1, squares; 7, circles; and 16, triangles) analyzed on different chip types (CM4, black; GLC, red; and C1, green). Each data point represents the mean and standard deviation of at least four independent measurements. The blue dotted lines represent the isoaffinities. See Table 2 for the kinetic parameters.

**Table 2**
Chip-dependent kinetic analyses of Ag1 binding amine-coupled mAbs. Biacore data were generated via both kinetic titration and classical injection methods and ProteOn data were collected via both kinetic titration and one-shot methods. The parameter values represent the mean (and standard deviation) of *n* independent experiments.

| MAb No. | Instrument | Chip | $10^{-5} \times k_a$ ($M^{-1}$ $s^{-1}$) | $10^3 \times k_d$ ($s^{-1}$) | $K_D$ (nM) | *n* |
|---|---|---|---|---|---|---|
| 1 | Biacore | C1 | 9.3 (0.2) | 0.70 (0.03) | 0.75 (0.04) | 6 |
| 7 | Biacore | C1 | 9 (1) | 13 (1) | 14 (3) | 6 |
| 16 | Biacore | C1 | 1.5 (0.2) | 4.1 (0.4) | 28 (4) | 6 |
| 1 | ProteOn | GLC | 7 (1) | 0.70 (0.05) | 1.0 (0.2) | 6 |
| 7 | ProteOn | GLC | 6 (2) | 12 (1) | 19 (5) | 6 |
| 16 | ProteOn | GLC | 1.0 (0.1) | 4.1 (0.5) | 40 (7) | 6 |
| 1 | Biacore | CM4 | 2.2 (0.1) | 0.59 (0.03) | 2.7 (0.2) | 4 |
| 7 | Biacore | CM4 | 2.5 (0.6) | 12 (1) | 50 (10) | 7 |
| 16 | Biacore | CM4 | 0.55 (0.09) | 3.8 (0.6) | 70 (20) | 4 |

self-sandwiching signal. Neither Ag nor any of the solution mAbs bound the negative control mAbs and the blank spot, increasing our confidence that Ag-binding and IgG-sandwiching responses were specific elsewhere in the assay. Fig. 7B shows the final binning matrix obtained (using the same classification methodology as applied to the ProteOn binning data) from the simultaneous analysis of 16 tips on the Octet QK384, each coupled with a different mAb and overlays it with the corresponding binnings obtained on the ProteOn (as defined by the black boxes).

*Using the ProteOn as a 36-ligand array in epitope mapping*

The goal of this portion of the study was to epitope map a large panel of mAbs onto their target Ag (a 15-kDa monomer). We used reagents that were unrelated to those described in the above kinetics and binnings examples. We custom-ordered a library of biotinylated peptides (biotides) and arrayed them onto individual spots of a ProteOn chip to provide a platform for screening mAbs in succession, regenerating the immobilized biotides after each mAb. To capture a different biotide per spot via preimmobilized streptavidin, we modified the protocol that was used to array ligands by amine-coupling (see Materials and methods). None of the mAbs bound nonspecifically to the unmodified chip or the biotin-blocked streptavidin surfaces, so either type of interspot was suitable as a reference surface; we screened mAbs along the horizontal flow path, but directing them along the vertical flow path would have given an equivalent result. Six mAbs were epitope-mapped per hour in a one-on-many format based on a 10-min cycle time that included a mAb injection and a regeneration step.

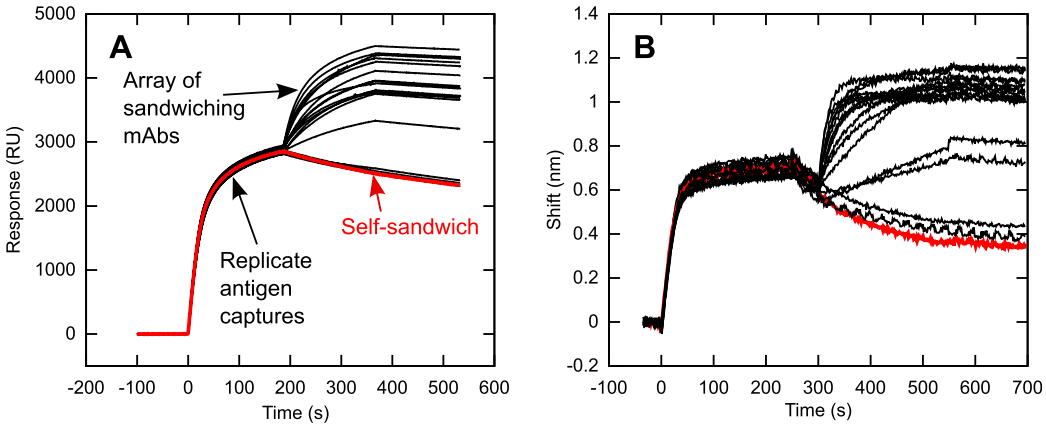


**Fig.6.** Classic sandwich binning on (A) the ProteOn when used as a 36-ligand array and (B) the Octet QK384. Eighteen rounds of binning on (A) a single spot coupled with an IgG or (B) a single tip coupled with the same IgG; in both cases the "self-sandwich" is highlighted in red.

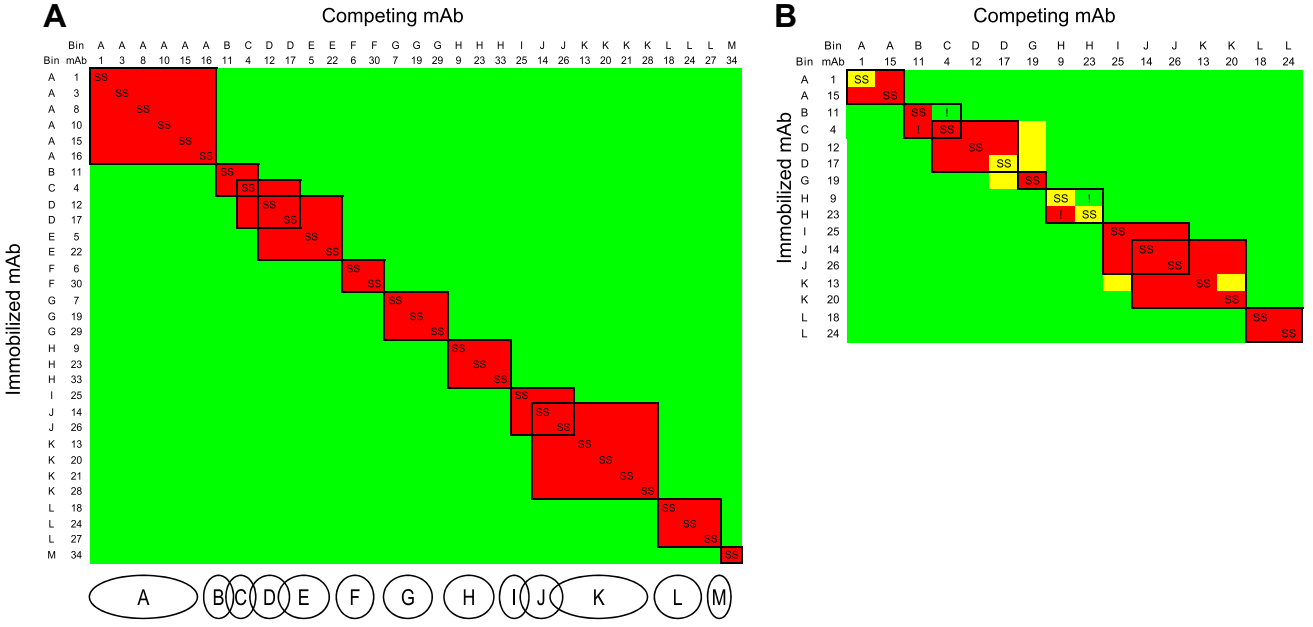


**Fig.7.** (A) Pairwise binning matrices for 31 mAbs obtained in a single experiment on the ProteOn. Sandwiching and nonsandwiching pairs are shown in green and red, respectively, with "SS" indicating the "self-sandwiching" controls. The Venn diagram provides an alternate view of the bins (A–M) that are also listed across and down the matrix. The isolated circles represent bins that sandwiched with all other bins, suggesting that these epitopes were remote from others, and the intersecting circles represent bins that blocked other bins, consistent with their epitopes being similar but not identical to one another. (B) Full pairwise binning matrix for 16 mAbs on the Octet QK384 superimposed on the binnings determined for these interactions via ProteOn (as defined by the black boxes). Green and red cells represent sandwiching and blocked pairs of mAbs, respectively. Yellow cells represent intermediate (ambiguous) responses. "!" represents a conflicting result that was dependent on the order of addition and thus introduced an asymmetry into the matrix.

The left panel in Fig. 8 shows the binding responses for five mAbs (A–E) that bound distinct epitopes as judged by them each mapping to different biotides. The data look smooth because the signals are several hundred times larger than the instrument noise. The binding responses for duplicate injections of mAb "A" analyzed several hours apart were highly reproducible, confirming that all surfaces tolerated multiple rounds of regeneration. To validate these results on an orthogonal biosensor platform and illustrate the mapping throughput that could be achieved in an unattended mode, we screened a smaller set of interactions on the Octet QK384. The results from this assay are shown in the middle panel of Fig. 8. The right panel in Fig. 8 shows the results obtained from a similar epitope mapping assay performed on a Biacore using the Biotin CAPture kit to allow for the reversible and highly reproducible immobilization of a large number of biotinylated ligands in a single assay [11].

## Discussion

### *Single spot analysis on the ProteOn 36-ligand array*

Our results demonstrate that kinetic titration enables the simultaneous analysis of 6-fold more analyte/ligand pairs than the conventional one-shot kinetics because it interrogates single spots rather than whole channels. Unlike one-shot kinetics, kinetic titration does not rely on a ligand being uniformly distributed along a strip, freeing the assay of undesirable artifacts, such as a gradient of immobilization levels along a strip (typically, we have found that a ligand is depleted 3% along a strip on immobilization – see Section "Materials and methods") or differential regeneration at different analyte concentrations. To determine the reliability of single spot analysis on the ProteOn, we studied the kinetics of a 13-kDa monomeric antigen interacting with various mAbs using

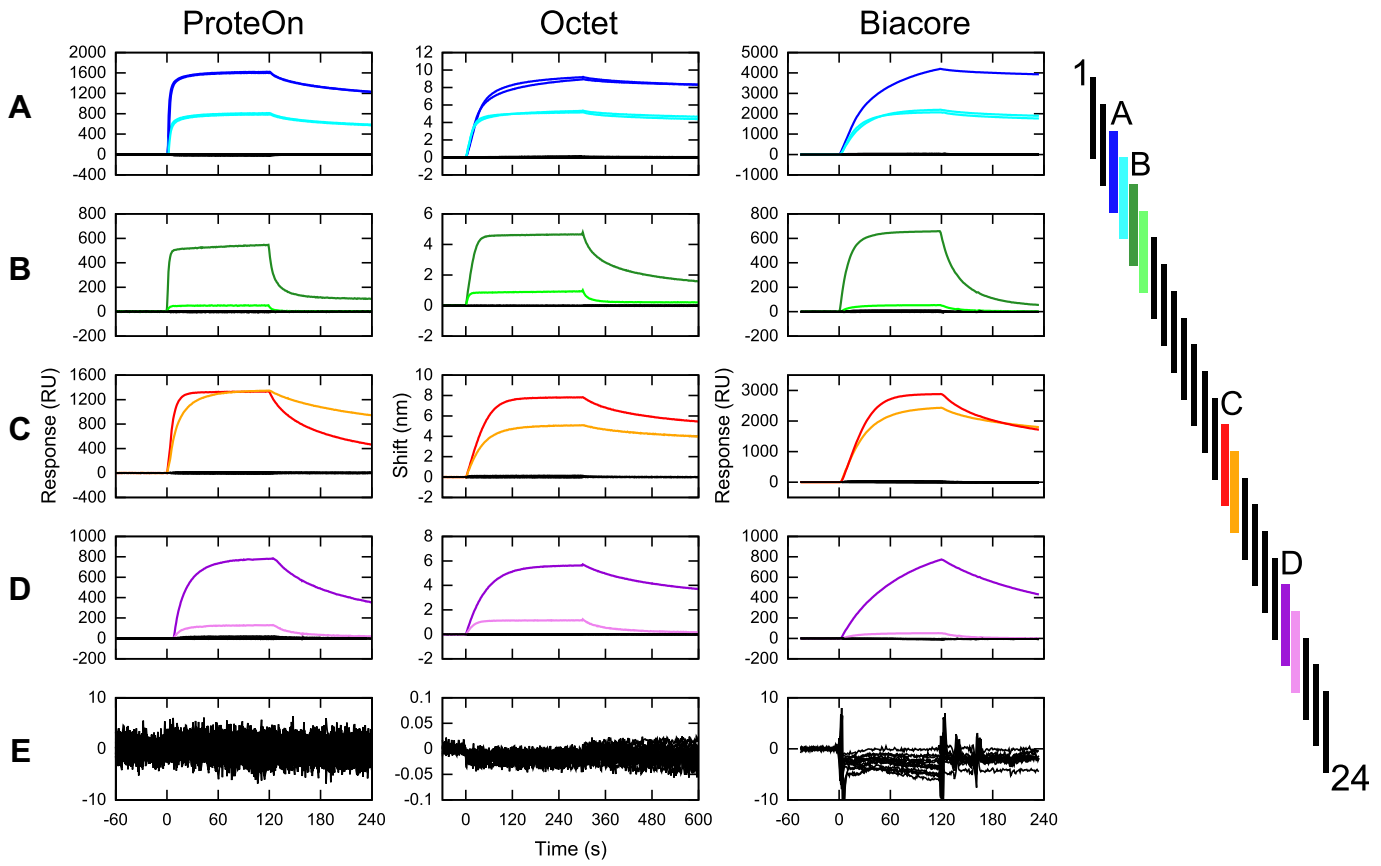


**Fig.8.** Epitope mapping a panel of 16 mAbs against a biotide library captured via streptavidin surfaces on three different biosensor platforms, ProteOn XPR36 (left), Octet QK384 (center), and Biacore 2000 (right). Within the test panel, we identified mAbs that mapped to four different epitopes; panels A–D represent an example of each one. Some mAbs did not bind any biotide (panel E) presumably because they bound to a conformational epitope.

two different SPR biosensors, different chip types, different immobilization chemistries, and different kinetic methodologies. We found excellent "within-instrument" precision of the rate constants determined on the ProteOn via two methodologies, namely kinetic titration or one-shot kinetics (Fig. 2). Similar within-instrument precision was found on the Biacore when we compared the use of kinetic titration and classical injection methodologies on amine-coupled ligands, as reported by other investigators [7]. We also found excellent precision in experiment-to-experiment reproducibility for two independent capture assays performed in a kinetic titration mode on the Biacore (see Table 1). Since the Biacore 2000 instrument has significantly fewer ligand surfaces than the ProteOn (4 and 36, respectively), we utilized a capture approach for the Biacore assay in order to increase the number of ligands that could be investigated per unattended run. Typically capture methods are restricted to ligand panels that are amenable to a universal capture reagent and tolerant of multiple rounds of regeneration; however, these methods can be used with crude antibody samples since the capture step serves as an on-line purification step [4,12,13]. Recently, a similar capture-based technique has been employed on the ProteOn [14].

Despite the use of different immobilization chemistries, the Biacore assays returned very similar results (compare Tables 1 and 2 for mAbs 1, 7, and 16). However, across instruments we observed some statistically significant differences in the absolute values of $k_a$, which was systematically higher on the ProteOn, resulting in affinities being tighter on the ProteOn (Table 2). Our results suggest that this discrepancy was caused by the use of different chip types; the ProteOn's alginate-based chips gave kinetic parameter values that were closer to Biacore's flat (matrix-free) C1 chip than the more commonly used carboxymethyldextran-based Biacore chips (CM4 and CM5).

Arraying 36 unique ligands on the ProteOn takes approximately 3 h and cannot be fully automated because the activation reagents must be mixed manually immediately prior to use; our method incorporated six activation steps that each required hands-on time because the current software lacks functionality analogous to the "transfer and mix" injection command implemented in Biacore systems that enables unattended immobilizations. A 36-ligand array provides a diverse platform that can expedite screening of a single analyte without any regeneration and if the ligands can be regenerated, as they were in our examples, analyte-binding cycles can be replicated, as we demonstrated in our kinetic example, or multiple analytes can be investigated, as we demonstrated in our binning and mapping examples. Not having to regenerate allows the study of sensitive ligands where suitable regeneration conditions may be unknown and removes the need to scout for regeneration conditions during assay development. In principle, this methodology can be applied to other multiplexed many-on-many biosensors such as the Biacore 4000, where kinetic titration would enable, for example, the study of one-on-16 or four unrelated one-on-four panels.

### *High-throughput binning and mapping allows for detailed epitope characterization*

Increasing the number of pairwise interactions that can be addressed per assay improves the resolution of an epitope binning study and increases the chance of discovering mAbs with unique epitopes. In our example, we identified 13 bins by competing

31 mAbs against one another. Six of these bins (A, F, G, H, L, and M) did not overlap with any other bin, whereas other bins were very similar to one another, such as D and E, which were discriminated only by their interaction with a single mAb that was the sole member of bin C. From a functional perspective, only one bin showed any effect in a cell-based assay; all mAbs in bin H were active in a cell-based assay. While the Ag used in the binning portion of this study has been implicated as a therapeutic target, its native binding partner is unknown, so identifying mAbs with diverse epitopes increases the chance of discovering a bin or bins that correlate with *in vivo* efficacy.

The binning data from the ProteOn and Octet generally agreed, with the few discrepancies being mostly attributable to a higher signal-to-noise ratio on the ProteOn that facilitated a more definitive interpretation of the data. Some of the ambiguous responses in the Octet binnings (yellow cells in Fig. 7B) were due to mAbs that bound Ag with poor signals (9 and 19), mAbs with abnormally large self-sandwiches (1, 9, and 17), mAbs that did not regenerate sufficiently (12, 13, and 17), or mAbs that were unaffected by regeneration (23). The Octet Red384 is a recent upgrade to the Octet QK384 that has higher signal-to-noise which may minimize inconclusive binnings. We exhausted the full square reactivity pattern matrix of interactions because an ambiguous result in one orientation may yield an unambiguous result in the opposite orientation. Furthermore, we have encountered other Ag/mAb systems that bin asymmetrically, indicating that the order of addition of mAbs to some Ags may be important; in the current example, a few asymmetries were apparent in the Octet data set (Fig. 7B). If the ability of two antibodies to sandwich is ambiguous in both orientations or nonsymmetrical binning is suspected, follow-up assays on fewer interactions can be performed to disambiguate the binning result.

Sandwich binnings enable mAbs to be discriminated into groups based on whether or not a pair of mAbs can bind simultaneously to the native Ag, but bins are relative to one another and do not identify the precise epitope at the amino acid level. In contrast, epitope mapping employs nonnative modified forms of the Ag, such as mutations, truncations, or other fragments (as used in the current study) to pinpoint the amino acids on the Ag that contribute to an epitope of interest. Binnings and mappings are therefore complementary tools in defining epitopes. Combining the information from both methods builds a more complete description of epitopes and their relative impact on one another and aids in identifying conformationally sensitive antibodies, i.e., those that bind the full-length native Ag, but do not bind any biotides (Fig. 8E).

### *Throughput considerations*

For users of the ProteOn, combining the 36-ligand array with kinetic titration offers an alternative to the one-shot kinetic method, which may be advantageous in antibody drug discovery applications where the immobilized ligands are often large panels of IgGs (immobilized to avoid avidity effects) and the analyte panel comprises a small number of antigens (typically the target monovalent antigen and perhaps closely related antigens or the same antigen from different species). When selecting the optimal kinetics assay format on the ProteOn a number of factors need to be taken into consideration; for example, the cost of the consumables, the number of unique interactions that can be characterized in a given time, and the number of unique interactions that can be investigated per unattended run. Table 3 shows a detailed comparison of these factors for the one-shot method and our single spot 36-ligand array method when the ligands are directly coupled to the sensor surface.

Single spot analysis on the ProteOn enables 36 unique analyte/ligand interactions to be studied simultaneously, which rivals the number that can be measured simultaneously by many other commercial biosensor platforms. However, other factors, such as the autosampler capacity, hands-on time, and assay flexibility must be taken into consideration when comparing the throughput of platforms and their suitability for a given application. For instance, while the Biacore 4000 enables the simultaneous investigation of 16 interactions, its rack hotel can accommodate ten 96-well or 384-well microplates and ten 24-well (4 ml/well) trays, in contrast with the ProteOn's autosampler which can hold two 96-well microplates. Therefore, the expanded autosampler capacity of the Biacore 4000 allows for longer unattended runs with a larger total number of interactions being investigated per run. Another important factor is the hands-on time needed to prepare an assay, which includes preparing the instrument, immobilizing the ligands, and dispensing the samples. For example, while SPR imaging-based biosensors can monitor the binding of one analyte over hundreds of ligands simultaneously, the routine implementation of these technologies has been hindered by the lack of a reproducible printing method for immobilizing ligands and an inability to immobilize proteins from low-concentration crude mixtures such as hybridoma supernatants. Recent developments

**Table 3**
Comparing the throughput and practical aspects of one-shot kinetics and array-based kinetic titration.

| Method | One-shot kinetics | Array-based kinetic titration |
|---|---|---|
| No. of chips required to prepare 36 unique surfaces (only one chip can be docked at a time per instrument) | 6 | 1 |
| Time to prepare surfaces per chip[a] | 1 h | 3 h |
| No. of unique ligands per chip | 6 | 36 |
| Time per analyte cycle[b] using a 3-min association phase and a 15-min dissociation phase | 25 min | 65 min |
| Time for a buffer blank cycle | None (included "in-line" as part of the analyte cycle) | 65 min |
| No. of unique interactions per analyte cycle | 6 | 36 |
| Total time to generate binding data for one analyte interacting with 36 unique ligands | 9 h (requires hands-on time to prepare each chip) | 5 h |
| Total No. of unique analytes that can be injected per docked chip (as limited by the capacity of the autosampler and contingent on the successful regeneration of all ligands)[c] | 30 | 6 |
| Total No. of unique analyte (A) × ligand (L) pairs that can be analyzed unattended per chip (with regeneration) | 30A × 6L = 180 | 6A × 36L = 216 |
| Total time needed to collected all the kinetic data for the formats described in the above row | 15 h | 11 h |

[a] Includes the time needed to normalize the chip, flush the instrument, immobilize the ligands, and stabilize the baseline with up to five 30-s injections of buffer.
[b] Incorporates an additional 5 min needed for pre/postinjection needle washes and baseline stabilization as defined by the default parameters for each "Analyte" (maximum) quality injection.
[c] Based on the use of two deep-well 96-well plates (2.0 ml/well) to accommodate the regeneration and wash buffers.

in continuous-flow spotting methods are providing a promising alternative to the conventional pin-spotting approach for microarray fabrication and are generating interest in SPR imaging-based biosensors [15]. The Octet QK384's microfluidic-free dip-and read technology requires minimal maintenance compared to microfluidic-based platforms which require specialized cleaning and preparation routines such as sanitization, desorption, and priming. The Octet QK384's ability to perform immobilizations simultaneously on 16 sensors on-line, or in a larger batch off-line, makes it an appealing high-throughput platform. It can accommodate 96 disparate directly coupled ligands per assay or more ligands if a capture method is employed, and despite its addressing of samples from open microplates, we have accomplished unattended run times of 13 h with appropriate plate volumes and well-depth settings for binning and mapping applications (data not shown). Since each analyte/ligand pair and their buffer conditions are independent of one another, diverse panels of interactants can be studied in a given run making this a very flexible platform.

The increased throughput and utility of array-based biosensors require appropriate software support to manage and analyze the volume of data that they generate. The current analysis software on the ProteOn does not support the analysis of kinetic titration data and requires the data to be exported and analyzed in an independent data analysis program such as BiaEvaluation, which is only available to Biacore users.

*Comparison with Biacore*

As new biosensor technologies become available and existing ones evolve, it is important to validate their performances by comparing them directly to well-established technologies such as Biacore, which has been an industry standard since the 1990s and for which data from large multiuser benchmark studies are available [16]. In general, the correlation between the ProteOn- and the Biacore-derived affinity measurements and the excellent agreement of the epitope mapping results suggest that the former may be an appropriate substitute for the latter in these types of assays. This point is reinforced by considering the greater than 10-fold difference in time required to complete the epitope mapping on the two platforms; having arrayed 36 ligands on the ProteOn in 3 h, one mAb could be mapped every 10 min. Mapping 12 Abs against 36 biotides was accomplished in 5 h on the ProteOn, inclusive of the immobilizations, while the equivalent mappings on the Biacore 2000 using the Biotin CAPture kit would have taken longer than 2 days. This difference in time would be reduced on a higher throughput Biacore platform such as the Biacore 4000.

As multiplexed biosensor technologies have become available, the diversity of possible experimental configurations has increased. Which biosensor platform is ideal for a given application may differ from application to application and with the number of unique analyte/ligand pairs that need to be investigated per project. This work has shown that, while current multiplexed technologies can be used in the same application areas, there are often trade-offs in the available assay configurations and throughput from platform to platform. We have demonstrated that expanding the ProteOn into a 36-ligand array greatly increases its throughput and versatility in a variety of applications.

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