Grading the commercial optical biosensor literature—Class of 2008: 'The Mighty Binders'

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Optical biosensor technology continues to be the method of choice for label-free, real-time interaction analysis. But when it comes to improving the quality of the biosensor literature, education should be fundamental. Of the 1413 articles published in 2008, less than 30% would pass the requirements for high-school chemistry. To teach by example, we spotlight 10 papers that illustrate how to implement the technology properly. Then we grade every paper published in 2008 on a scale from A to F and outline what features make a biosensor article fabulous, middling or abysmal. To help improve the quality of published data, we focus on a few experimental, analysis and presentation mistakes that are alarmingly common. With the literature as a guide, we want to ensure that no user is left behind. Copyright © 2009 John Wiley & Sons, Ltd.

What's that old saying?—oh yeah, something like 'If you give a man a fish, he can eat for a day. But if you teach a man to do a biosensor experiment correctly, he can buy all the damn fish he wants'. For years we have taken an active approach to educating users, as well as non-users, about how to properly design, execute and analyse biosensor experiments. Unfortunately, based on our annual review of the literature we still have a lot of work to do. Only a minority of users know how to run even basic experiments correctly. And based on what's published, there is still a general perception that whatever data comes out of the biosensor must be biologically relevant and should be published. Nothing could be farther from the truth.

Depressingly, and in some ways frighteningly, the biosensor user community is not the only scientific field in the need of some reform, regulation and education. Take as an example the recent report from the National Academy of Sciences on the state of forensic science:

''Badly Fragmented' Forensic Science System Needs Overhaul. 19 February 2009—A US congressionally mandated report from the National Research Council finds serious deficiencies in the nation's forensic science system and calls for major reforms. Mandatory certification programs for forensic scientists are currently lacking, as are strong standards and protocols for analysing and reporting on evidence.

Many professionals in the forensic science community and the medical examiner system have worked for years to achieve excellence in their fields, aiming to follow high ethical norms, develop sound professional standards and ensure accurate results in their practice. But there are great disparities among existing forensic science operations. The disparities appear in availability of skilled and well-trained personnel; and in certification, accreditation and oversight. This has left the forensic science system fragmented and the quality of practice uneven. These shortcomings pose a threat to the quality and credibility of forensic science practice and its service to the justice system.'

Replace the words 'forensic' and 'justice system' with 'biosensor' and 'biological sciences' and you would have a pretty accurate depiction of what our user base suffers from as well. But until one (or both) of us is (are) elected to the National Academy of Sciences (which seems less likely now than ever after writing this review), we figure we will continue our role of educating a field which is in desperate need of standardization.

So grab your pole and put on some sunblock because you're about get schooled on the Moby Dick, 20 000 Leagues Under the Sea, Finding Nemo biosensor adventure of a lifetime. You're gonna need a bigger boat.

ROLL CALL

First let's get everyone on the same page by summarizing the year's literature. We found 1413 articles published in 2008 that describe biosensor-based experiments. In the reference list, this collection is divided into several sections: books, editorials and general reviews about biosensors (1–23); theory, methods development and instrument comparisons (24–42) and instrument- and application-specific articles (43–1413).

A review article by Comley is particularly worth reading because it summarized the results from recent interviews conducted as part of a biosensor market report (5). He compiled the survey's finding about interviewees' familiarity with biosensor

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manufacturers and their products, user concerns about the different instruments, the biological systems examined and the type of information sought by users, and the technology's perceived challenges (i.e. factors that prevent it from becoming more widely adopted). In addition, Comley provides snapshots of the various label-free vendors and the platforms each offers.

It seems like every year someone writes a general article describing surface plasmon resonance (SPR) experiments. This year it was De Crescenzo *et al.*'s turn (6). They reviewed the physics of SPR, described Biacore's dextran layer, outlined methods to reduce experimental artefacts (e.g. buffer preparation, ligand immobilization methods and interaction stoichiometry issues) and discussed the basics of kinetic analysis, including important processing steps and considerations during model fitting.

As illustrated in References 29–37, several biosensor users continue to push the envelope in assay optimization and technology development. A number of researchers reported new approaches to tethering ligands to sensor surfaces (29,30,32–35). For example, Gale and collaborators (admittedly, we are some of them) developed a continuous-flow microspotter to be used with SPR imaging instruments (32–35). Unlike traditional pin-spotting techniques, this in-solution spotter does not require pure, highly concentrated ligand solutions nor does the ligand surface need to be dried after immobilization. We expect this approach will resolve many of the concerns surrounding array platforms and make these instruments useful for a wide range of applications.

And, as the number and variety of optical biosensors increases, it can be daunting to understand the benefits of the different platforms. Therefore, reports of comparative studies (like References 38–42) are informative articles for the general biosensor community.

The remainder of the reference list relates to specific instruments or applications. We found articles describing work performed using instruments offered by 30 manufacturers (Table 1). These instruments employ SPR (in either standard (43–1338) or array formats (1339–1391)) or alternative optical detection methods (1392–1413). The reference list is further divided by manufacturer and, since the number of Biacore references is so large, these articles are grouped by biological application.

In reviewing this collection, we are encouraged by authors who put a lot of work into their experiments. For example, several research groups used multiple immobilization chemistries (e.g. References 372,947) and prepared surfaces of different ligand densities (267,452), tested their interactions in both orientations (593) and used different assay formats (101), and carefully examined the responses obtained from the reference surface (1014). Notably, Bravman *et al.* emphasized the care required when choosing the pH for immobilization (1341). This group nicely demonstrated that the pH which produced the highest surface density was in fact not necessarily optimal for ligand activity. Call us old school, but we think it never hurts to be reminded about the fundamentals of a good biosensor experiment.

HONOR ROLL

The 10 articles we chose to spotlight this year exemplify the power of biosensor technology and the tenacity of some great users. These are the summa cum laudes of SPR; they give us hope that perhaps in the not-so-distant future everyone will produce high-quality biosensor data.

The 10 articles, summarized in the box below, describe significant developments in biosensor applications, report well-performed, in-depth experiments and include easy-to-interpret figures of data, and/or provide insights about where this technology is heading with respect to improved throughput and sensitivity. Consider each of these papers required reading.

Advancing Applications

Giannetti *J Med Chem* 51:574.
Chavane *Anal Biochem* 378:158.
Christensen *J Aller Clin Immun* 122:298.

Rigorous Analyses

Souphron *Biochemistry* 47:8961.
Kalyuzhniy *Proc Natl Acad Sci USA* 105:5483.
Wang *J Virol* 82:10567.

Technology Validation

Abdiche *Prot Sci* 17:1326.
Abdiche *Anal Biochem* 377:209.
Beusink *Biosens Bioelectron* 23:839.
Rich *Anal Biochem* 373:112.

Giannetti *et al*. Surface plasmon resonance based assay for the detection and characterization of promiscuous inhibitors. *J Med Chem* 51: 574–580.

Giannetti *et al.*'s detailed characterization of promiscuously binding compounds emphasizes how biosensors can impact both rigorous small-molecule kinetic analyses and higher-throughput screening assays (923). As these researchers noted, too often compounds that appear promising in biochemical screens are in fact false positives and biosensor analyses help weed out these non-specific binders.

For example, Figure 1A shows the responses for a positive-control compound binding to an immobilized target. This well-behaved compound produced concentration-dependent responses that could be described by a simple interaction model. These data confirmed the target was active on the chip surface. Not all compounds, however, produce such easily interpretable responses. For example, non-stoichiometric compounds produce responses that are larger than the expected R_{max} (Figure 1B top left panels) while superstoichiometric compounds display responses of more than five times the R_{max}, often without any indication of surface saturation (Figure 1B bottom left panels). The right panels of Figure 1B depict examples of compounds that are well-behaved at lower concentrations but then suddenly show unexpectedly large responses at slightly higher (concentration-dependent aggregators) and compounds that become well-behaved in the presence of detergent.

But, as these researchers demonstrated, testing a compound library against only one target is not enough to determine which compounds to flag as bad binders. Some compounds behave differently depending on the target. Figure 1C shows the responses for one compound tested against seven targets and an unmodified surface. To some targets this compound displayed little or no binding while it showed intermediate but non-stoichiometric binding or superstoichiometric binding to other

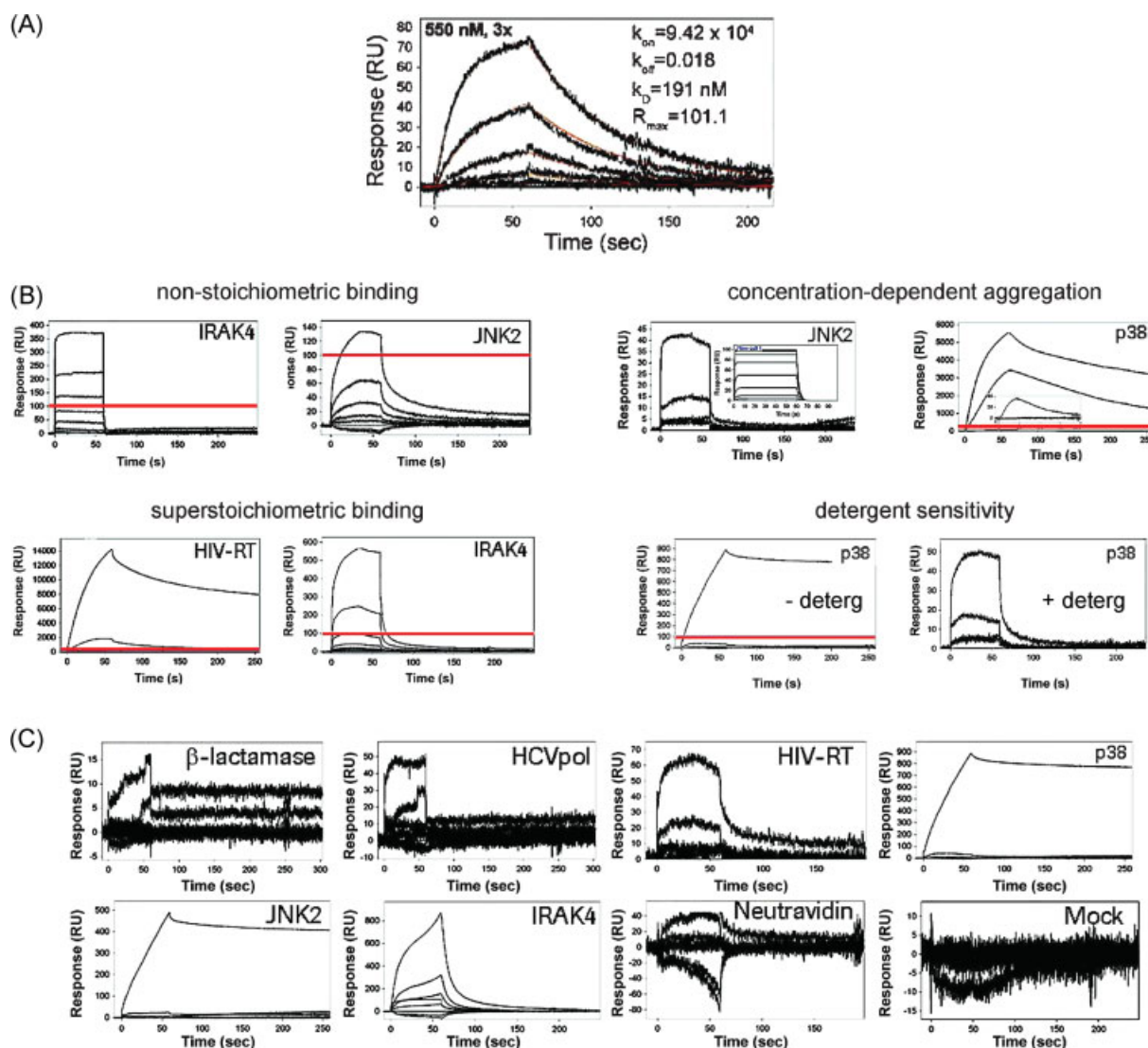**Table 1.** Commercial optical biosensor technologies

Manufacturer	Instruments	Website	References
Standard SPR platforms			
Analytical μ-systems	Biosuplar, Biosuplar-2, -3, -6	micro-systems.de	(43–52)
Biosensing Instruments	BI-2000, -2000G, -3000, -3000G	biosensingusa.com	(53–59)
DKK-TOA	SPR-20	dkktoa.co.uk	(41,60–62)
EcoChemie/Autolab	Esprit, Springle	ecochemie.nl	(63–89)
GE Healthcare/Biacore	Biacore, Bialite, C, J, Q, X, 1000, 2000, 3000, S51, T100, A100, X100	biacore.com	(29–31,36–39,90–1230)
IBIS	IBIS II	ibis-spr.n	(1231)
K-MAC	SPR *LAB*, SPRmicro	www.kmac.to	(1232–1236)
Microvacuum/Artificial Sensing	OWLS 110, OWLS 120	owls-sensors.com	(40,42,1237–1247,1407)
Moritex/Nippon Laser & Electronics	SPR 670 M, SPR-Cellia	moritex.co.jp	(41,1248–1258)
Nanofilm Technology	EP³SPR	nanofilm.de	(1143,1259,1260)
NanoSPR	NanoSPR 310, 321, 621	nanospr.com	(1261–1264)
NeoSensors Ltd.	IAsys+, IAsys Auto+	neosensors.com	(884,1265–1294)
Nomadics/Texas Instruments	SensiQ, Spreeta	discoversensiq.com	(1295–1308)
NTT-AT	Handy SPR PS-0109	ntt-at.com	(1139,1309–1311)
Optrel	Multiskop	optrel.de	(1312–1324)
Plasmonic Biosensor	Plasmonic Multichannel	plasmonic.de	(1325)
Reichert Analytical Instruments/Xantec	SR7000, SR7000DC	reichertspr.com	(1326–1332)
Resonant Probes	SPTM	resonant-probes.de	(1333–1337)
Thermo Scientific	SPR-1000	thermo.com	(1338)
Imaging SPR platforms			
Alphasniffer	BiOptix	alphasniffer.com	(1339)
Bio-Rad	ProteOn XPR36	bio-rad.com	(38,39,1340–1357)
GE Healthcare/Biacore	Flexchip	biacore.com	(32–35,1358–1362)
GWC Technologies/Toyobo	SPRimager II, SPR 100, MultiSPRinter	gwctechnologies.com toyobo.co.jp	(1363–1382)
Horiba/Genoptics	SPRi-Plex, SPRi-Lab+	genoptics-spr.com	(1383–1388)
IBIS	iSPR	ibis-spr.n	(1389,1390)
Plexera	PlexArray	plexera.com	(1391)
Non-SPR platforms			
Corning	Epic	corning.com	(40,1392–1395)
Farfield Sensors	*Ana*light Bio200, BioFlex, CrystaLight, 4D	farfield-group.com	(1273,1396–1408)
ForteBio	Octet Q, QK, Red, Red384, QK384	fortebio.com	(38,39,1409)
Maven Biotechnologies	LFIRE	mavenbiotech.com	(1410)
Silicon Kinetics	SkiPro	siliconkinetics.com	(1411)
SRU Biosystems	BIND	srubiosystems.com	(40,42,1412,1413)

targets. These data sets emphasize the need to check for binding to off targets and control/reference surfaces when screening compound libraries.

Giannetti *et al.*'s article serves two essential purposes. First, it outlines a systematic approach to identifying and classifying promiscuous binders. Second, it presents a number of data sets from poorly behaved compounds. Too often users think responses like these are good data. It is important for all users to recognize how data from promiscuous binders may look and to realize that these responses may reveal information about

stoichiometry, reversibility and changes in compound behaviour over a range of concentrations.

Now while it may be frustrating to chemists to hear that their exciting leads are junk, a major advantage of the biosensor is that it can confirm real hits and identify false positives. The sooner false leads are abandoned, the better for the project overall. Work like Giannetti *et al.*'s is particularly important as biosensor technology is increasingly used in fragment screening, where weak-affinity (but less well-characterized) compounds are screened at fairly high concentrations and in relatively high

Figure 1. Responses for well- and poorly behaved compounds. (A) Responses of a well-behaved compound can be described by a simple interaction model (red lines). The highest concentration tested, number of replicates and binding parameters determined from the fit are shown as insets. (B) Classes of poorly behaved compounds. In each panel, the expected R_{max} is indicated by the red line. (In the figure of concentration-dependent aggregation responses, the insets show responses that are more like what would be expected for each compound.) (C) Protein-specific promiscuity of one compound. Adapted from Reference 923 with permission from the American Chemical Society © 2008.

throughput. The ability to efficiently pick out both true binders and false positives is essential when reviewing the massive amounts of data generated in these screens. And although Giannetti et al. focused on small molecules, these types of non-ideal responses are observed in all types of biological interactions. We've added these terms for the different classes of promiscuous binders to our biosensor vocabulary list (see the end of this paper).

Chavane et al. At-line quantification of bioactive antibody in bioreactor by surface plasmon resonance using epitope detection. *Anal Biochem* 378: 158–165.
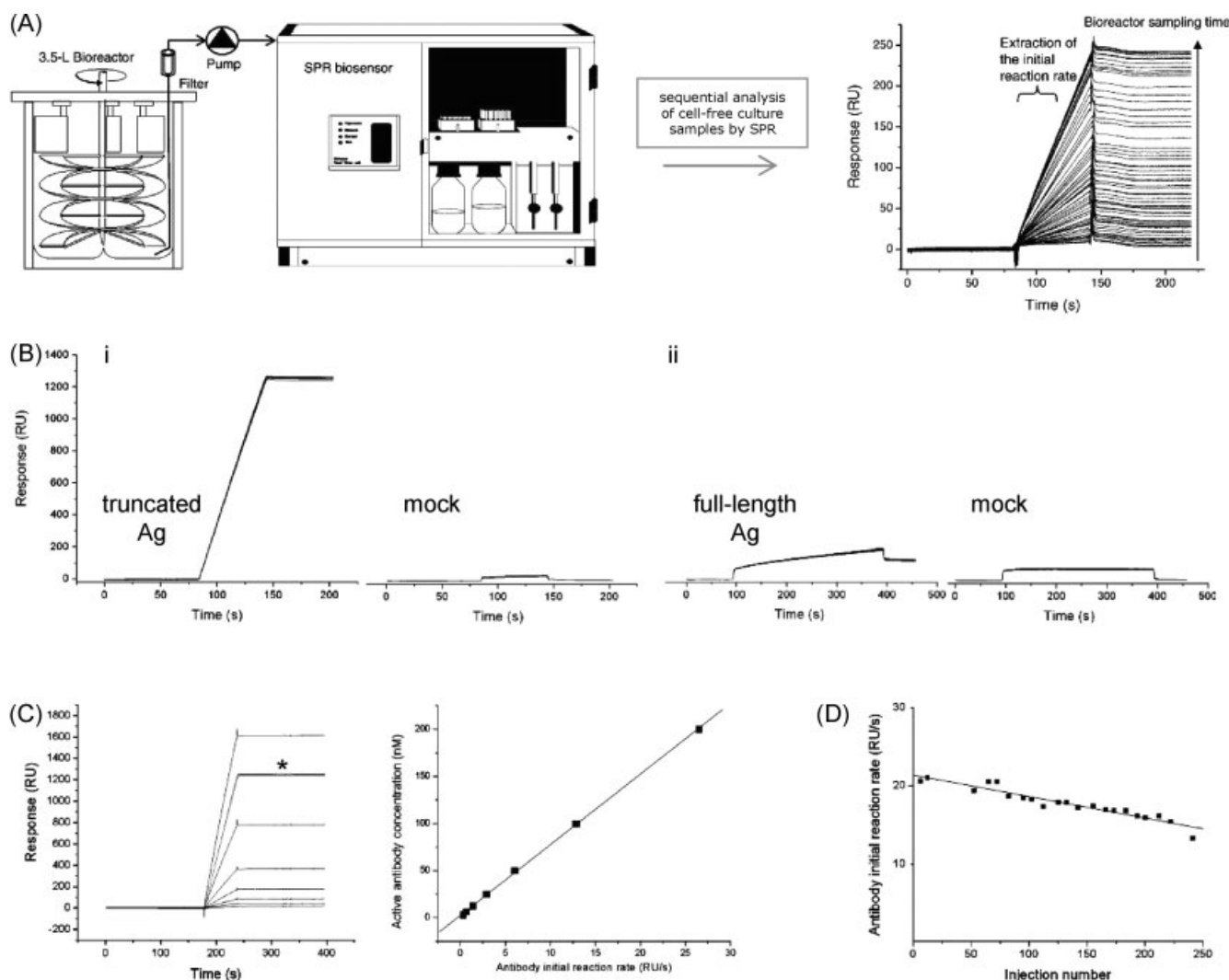
Chavane et al.'s report of a fully automated method to track antibody production highlights yet another unique application of biosensors (1212). This group devised a hands-off, at-line set-up (shown in Figure 2A) that tested crude medium from a bioreactor

every 10 h to quantitate the concentration of active antibody produced over 10 days.

Their biosensor approach has several advantages over more traditional production-tracking methods. Standard ELISA-like assays are labour- and sample-intensive, fluorescent assays require tags to engineered in and cleaved off and mass spectrometry assays measure the total production level but provide no information about activity. In contrast, the biosensor assay is label free, uses very little material and reveals both the amount and activity of the bioreactor product. But maybe best of all, its automation means the system can run unattended, even over Spring Break!

It should be noted that Chavane et al. first put a lot of effort into optimizing the assay off line. They needed to identify a suitable binding partner, appropriate immobilization chemistry and

Figure 2. At-line biosensor-based assay to track bioreactions. (A) Schematic of automated monitoring of antibody production. A pump continuously delivered filtered culture medium to s sampling vial in the biosensor. At set intervals, an aliquot of medium was diluted into running buffer using the Biacore 3000's sample transfer/mix functions and then injected across the sensor surface. The initial binding rate (measured at 90–120 s) in each sensorgram was used to calculate antibody concentration. (B) Off-line assay optimization. Ten replicate antibody injections across immobilized (i) antigen peptide and (ii) full-length antigen. Corresponding responses from mock surfaces are included in each panel. (C) Standard curve generation. Left: responses for a concentration series of purified antibody. Included are replicates for antibody from crude medium (highlighted by the asterisk). Right: calibration plot derived from the responses shown. (D) Trend plot of the responses for a standard antibody sample measured throughout the analysis. Adapted from Reference 1212 with permission from Elsevier Inc. © 2008.

regeneration solution and conditions that produced a mass transport-limited interaction. Figure 2B shows the responses for purified antibody binding to immobilized antigen (both full-length and peptide), as well as to mock surfaces. The low responses from the mock surfaces indicated the antibody bound specifically to the antigen surfaces. In addition, the overlay of 10 replicate antibody tests demonstrated the stability of both binding partners and the suitability of the regeneration condition. To induce mass transport-limited conditions that produce a linear association phase, Chavane *et al.* intentionally prepared high-density surfaces and used slow flow rates. They chose to use the peptide surface in follow-up experiments because it produced large, linear signals from which they could determine the initial binding rate, which correlates with antibody production level (Figure 2B, left panel).

Figure 2C shows the calibration data used to quantitate the bioproduct. This figure also includes a set of replicates from a crude antibody sample (highlighted by the asterisk). The similar shapes of the responses from purified antibody and the culture medium confirmed that other components in the medium did not bind to the ligand surface.

Figure 2D depicts the ageing of the ligand surface throughout the repeated binding/regeneration cycles over the 10 days of antibody culture. With this activity-monitoring information, Chavane *et al.* realized they needed to account for the surface's gradual loss in activity over time when calculating the bioreaction's output.

Two things make this paper required reading. First, the authors did a great job describing how the biosensor can automatically track culture growth over several days. Second (and maybe even

more important), several of the elements of their assay can be applied to biosensor-based concentration determination experiments in general.

Christensen *et al*. Several distinct properties of the IgE repertoire determine effector cell degranulation in response to allergen challenge. *Allergy Clin Immun* 122: 298–304.

Taking advantage of the biosensor's flexibility in assay design, Christensen *et al*. used both epitope mapping and kinetic assays to characterize a panel of 31 IgEs developed against major house dust mite allergen (Der p2) (358) (it might be mites).

With a two-stage epitope-mapping process, they investigated the antigen/antibody binding interface. First, they tested the ability of pairs of antibodies to bind Der p2 simultaneously (assay design illustrated in the top left panel of Figure 3A). If both antibodies bound, these researchers knew that the clones bound at separate sites in Der p2 (Figure 3A, top middle panel). If the second clone did not bind (e.g. Figure 3A, top right panel), they discovered the two clones bound at an overlapping site in Der p2. Then, using a related assay design (Figure 3A, bottom left panel), Christensen *et al*. screened wild type or mutant rDer p2 for binding to captured clones to identify residues in Der p2 critical for the Ab/Ag interaction (Figure 3A, bottom right panel). The pair-wise mapping revealed seven distinct antibody epitopes in Der p2 (Figure 3B, left panel) and the characterization of the variants indicated nine different binding patterns (Figure 3B, middle panel). Combining the information from these two complementary mapping experiments produced a three-dimensional model of the IgE epitopes in rDer p2 (Figure 3B, right panel).

Following up on the mapping experiments, Christensen *et al*. determined kinetic parameters for the various antigen/antibody pairs. The clones displayed a range of kinetic profiles (examples are shown in the top panels of Figure 3C), varied in their rDer p2 affinities by almost 30 000 fold, and were binned as high-, medium- or low-affinity binders (Figure 3C, bottom panel).

Christensen *et al*.'s coupled epitope mapping and kinetic approach illustrates the wealth of information that can be obtained using biosensors. In addition, their work incorporated features we look for in smart assay design when studying antibodies: (1) to avoid avidity effects, the antibodies were tethered to the surface rather than tested in solution and (2) by capturing the antibodies (rather than direct immobilization), the surface could be regenerated between binding cycles, thereby allowing different antibodies to be captured on the same sensor surface. Finally, this paper demonstrates the biosensor's efficiency. Most biosensors are automated, including the Biacore 3000 used by Christensen *et al*., and can test hundreds of clones in a single experiment.

Souphron *et al*. Structural dissection of a gating mechanism preventing misactivation of ubiquitin by NEDD8's E1. *Biochemistry* 47: 8961–8969.

Figure 4 depicts Souphron *et al*.'s examination of wild type and mutant NEDDs binding to ubiquitin and related proteins (279). These data sets are excellent examples of what we look for in an equilibrium analysis. Most importantly, in each panel the responses for every analyte concentration reach a plateau during the association phase. (This plateau indicates the interaction did in fact reach equilibrium and it was therefore appropriate to perform an equilibrium analysis.) Additionally, these researchers intentionally produced ligand densities that yielded relatively low analyte binding responses (R_{max} ~50 RU) and tested replicate analyte samples to demonstrate the reproducibility of their

experiments. And finally, the data shown in Figure 4 is easy to interpret. The data panels are scaled so we can quickly spot how well a particular NEDD protein is recognized by different ubiquitins, as well as which mutations decrease affinity or eliminate binding. Why can't all papers be this good?

Kalyuzhniy *et al*. Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes *in vivo*. *Proc Natl Acad Sci USA* 105: 5483–5488.

Wang *et al*. *In vitro* and *in vivo* properties of adenovirus vectors with increased affinity to CD46. *J Virol* 82: 10567–10579.
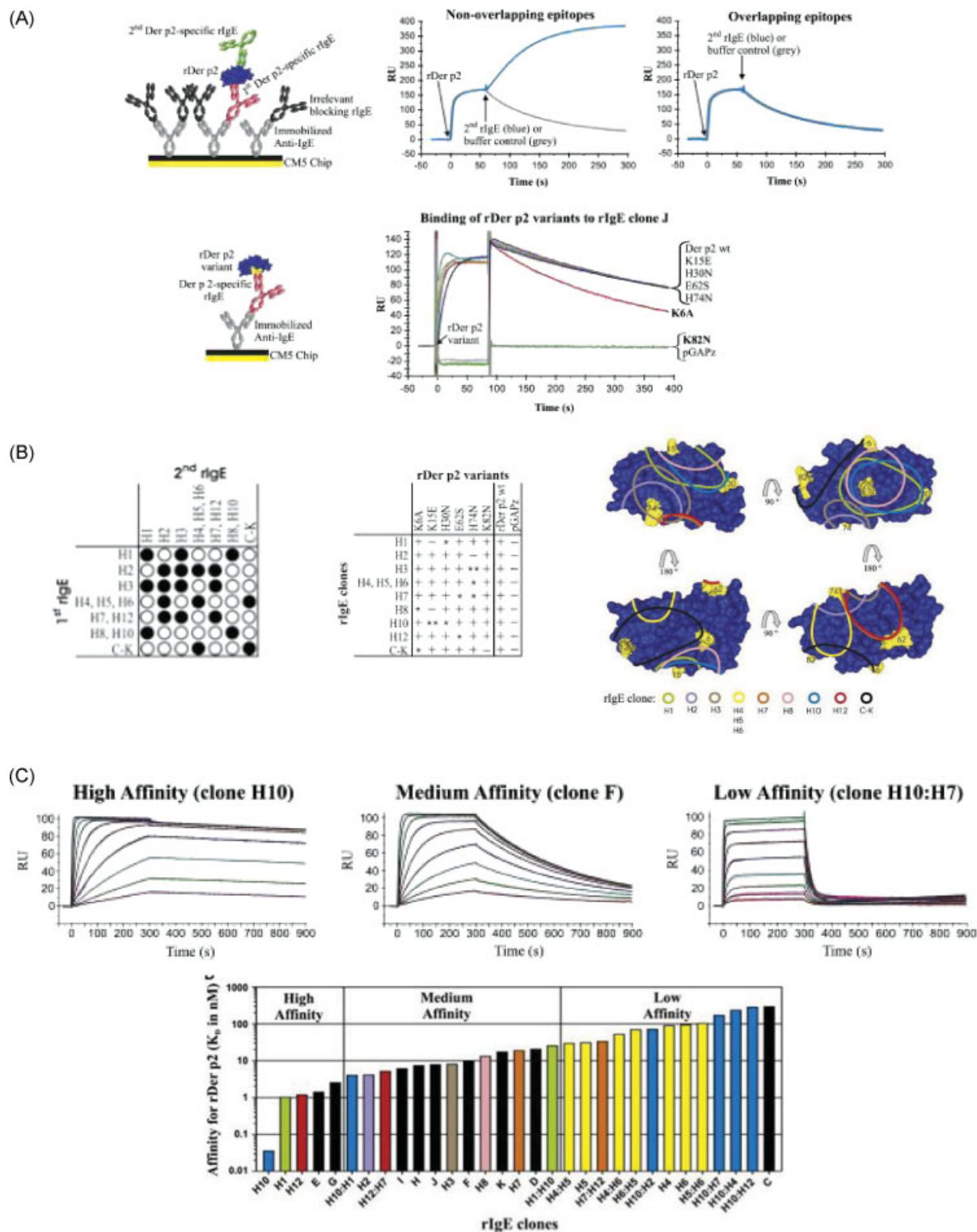
Work published by Shayakhmetov and co-workers exemplify well-performed kinetic analyses. In these studies of adenoviral interactions with blood coagulation factors and a receptor, the multivalent virus was immobilized (rather than tested in solution) to avoid avidity effects and surface densities were kept low to minimize other complexities. Analyte injection times were long enough for obtaining curvature in the association phase, the dissociation phases were monitored long enough to observe a decrease in response and a wide range of analyte concentrations were included in each experiment.

Figure 5 demonstrates the wealth of information available from a systematic kinetic characterization of related binding pairs. In this study, Kalyuzhniy *et al*. determined the adenoviral specificities and affinities for a subset of blood coagulation factors, which revealed the mechanism by which these viruses bind factor X and demonstrated the importance of the hexon protein in viral infection (172).

Especially noteworthy is Kalyuzhniy *et al*.'s use of what we call the 'short-'n-long' (SNL) approach to kinetic analysis of slowly dissociating complexes (an example is shown in the upper-left panel of Figure 5). Since this interaction is so stable, the dissociation phase must be monitored for a long time (in this example, >30 min) to collect enough decay in signal to obtain a reliable k_d. But these researchers recognized that there is enough decay information in the responses from the highest analyte concentration to determine k_d, so they truncated the dissociation-phase data-collection times for the lower analyte concentrations. (The SNL approach improves efficiency since it increases sampling throughput compared to the standard kinetic analysis format.) And, the overlay of the replicates confirmed the baseline was not drifting and the reagents were stable over the many hours required for each experiment. (We must repeat.)
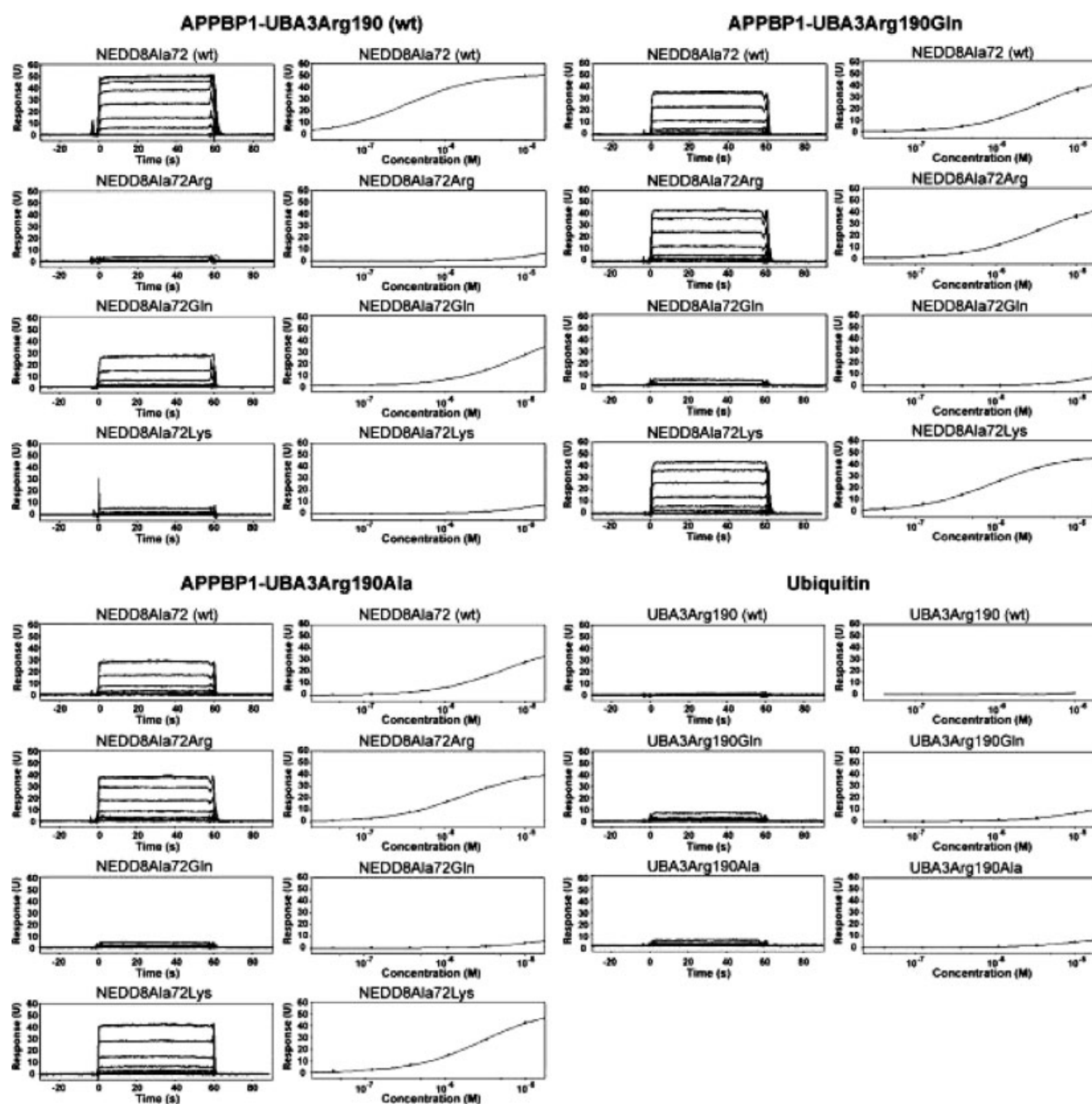
We were also happy to see Kalyuzhniy *et al*. fit all the data sets to a 1:1 interaction model, even those that display some complexity (i.e. the two panels highlighted with a star in Figure 5). Rather than invent an unlikely binding scenario to account for these secondary components, the authors realized the complexity most likely arose from biologically irrelevant artefacts. With the overlay of the data and fits, we can recognize how well the reported rate constants and affinities describe these particular interactions.

Figure 6 portrays the biosensor-based optimization of a transfer vector useful in gene therapies. Presuming that viral affinity for CD46, the receptor that some fibre knob-containing viruses use for cell invasion, correlates with infectivity, Wang *et al*. constructed a panel of mutant fibre knob particles and tested each against CD46 (Figure 6A). Several mutations produced particles that bound more tightly to CD46 compared to wild type, maintained their increased receptor affinity when incorporated into virions (Figure 6B), and indeed displayed greater *in vivo* transduction (1174).

Figure 3. Epitope mapping and kinetic characterization of Der p2-antibodies. (A) Assay designs (left) and responses (middle and right) for two complementary mapping experiments. The top panels depict the responses expected for the pair-wise analysis: a second IgE clone binding to an available site on IgE-captured rDer p2 (middle) and a second clone that would bind at the same site in rDer p2 as does the clone used to capture the antigen (right). In the analysis of antigen variants (bottom), the right panel depicts the responses for wild type and mutant rDer p2, as well as a negative control, pGAPz, binding to one IgE clone. (B) Summary of mapping experiments. Left: pair-wise analysis of 11 clones. Unfilled circles: rIgE pairs that can bind rDer p2 simultaneously; filled circles, rIgE pairs that cannot. Middle: analysis of six rDer p2 variants against 11 IgE clones. **, significantly altered; * somewhat altered; +, equal; -, no binding of the rDer p2 variant compared to wild type. Right: three-dimensional model of the clones' epitopes in rDer p2. (C) Top: example kinetic profiles of clones binding rDer p2 with high (left), medium (middle) and low affinity (right). Bottom: summary of the affinities determined for 31 clones, with specific colours corresponding to the epitope designations in the right panel of Figure 3B. Adapted from Reference 358 with permission from the American Academy of Allergy, Asthma and Immunology © 2008.

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Figure 4. Equilibrium analyses of wild type and mutant NEDDs binding to ubiquitin-like proteins and ubiquitin. In each data subset, responses for triplicate analyses of each concentration are displayed in the left panels while the right panels show the fits of the responses at equilibrium to a simple binding isotherm. Adapted from Reference 279 with permission from the American Chemical Society © 2008.
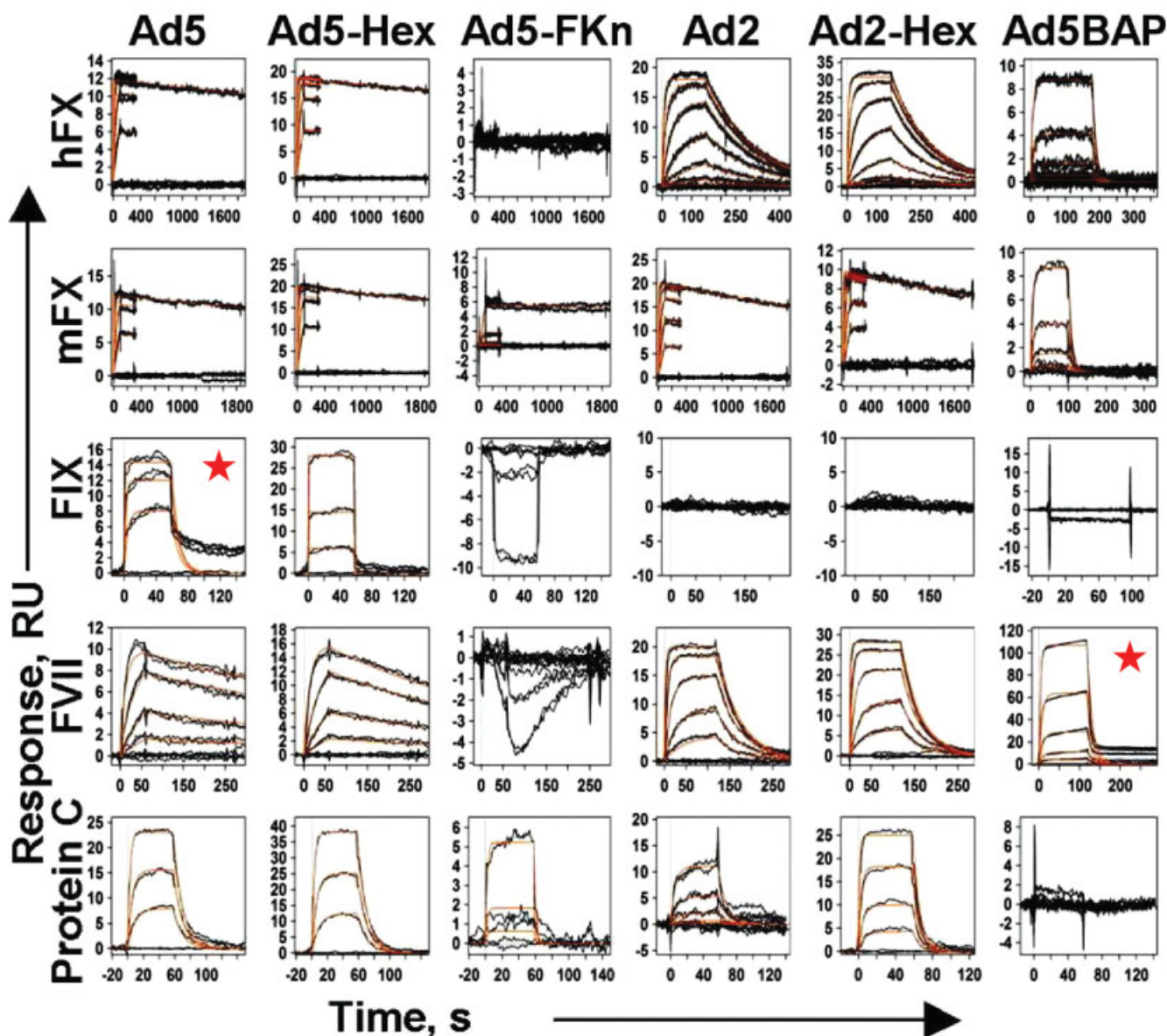
Wang et al.'s work illustrates how kinetic information can drive a research program. The differences in affinity between wild type and various mutant knob particles were due directly to changes in the off rate, so by comparing the dissociation rate constants Wang et al. identified which mutants to incorporate into virions.

From a technical standpoint, these biosensor studies are impressive for two reasons. First, Wang et al. were not intimidated by the 'bumpiness' (due to random, short-term noise) in binding responses that may be evident when working with low-density surfaces (e.g. the upper right panel of Figure 6A). Unlike biosensor users who prefer smooth-looking, large-intensity responses,

these researchers recognized that it is far better to publish data with these small deviations from ideality than to introduce artefacts that can arise at high surface densities. Second, concerned that a regeneration condition may damage the surface-tethered knob particles and/or virions, Wang et al. simply washed the surface with running buffer until the response returned to baseline (e.g. >45 min for some mutants). Sometimes doing nothing is something.

Abdiche et al. Probing the binding mechanism and affinity of tanezumab, a recombinant humanized anti-NGF monoclonal antibody, using a repertoire of biosensors. *Prot Sci* 17: 1326–1335.

Figure 5. Kinetic analyses of five blood coagulation factors binding to six adenoviruses and adenovirus capsid proteins. Red lines depict the fit of a 1:1 interaction model. Each panel depicts duplicate analyses of each analyte concentration. The panels highlighted with a star are referred to specifically in the text. Adapted from Reference 172 with permission from the National Academy of Sciences USA © 2008.
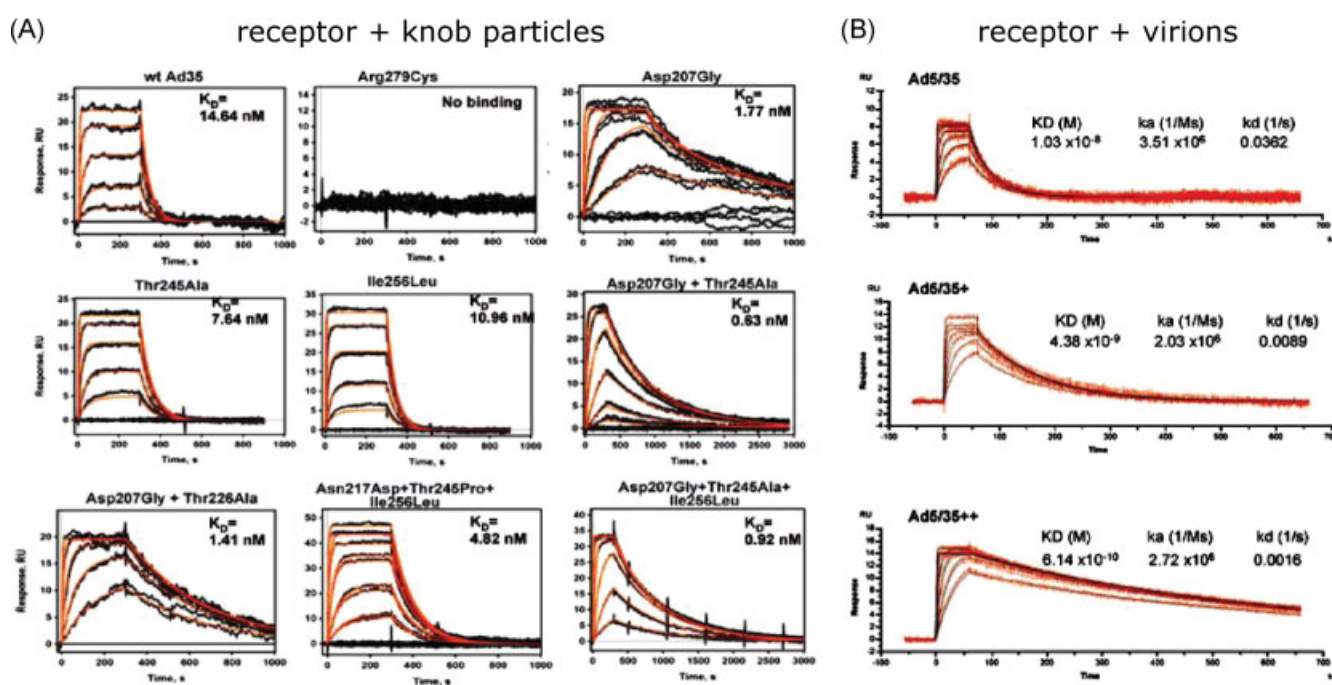
Abdiche *et al*. Determining kinetics and affinities of protein interactions using a parallel real-time label-free biosensor, the Octet. *Anal Biochem* **377: 209–217**.

As our options in biosensor technology multiply, it becomes increasingly important to evaluate the different instruments. Abdiche *et al*.'s side-by-side comparison of the GE Healthcare Biacore 3000's, BioRad ProteOn XPR36's and ForteBio Octet QK's detection systems, sampling methods and throughput, as well as the precision of the obtained binding parameters, demonstrate the advantages and disadvantages of these three platforms.

In one comparative study, Abdiche *et al*. characterized the interaction of the antibody tanezumab and human nerve growth factor (NGF) (39). This Ab/Ag pair is particularly difficult to work with using the biosensor because both binding partners are bivalent (NGF forms homodimers) and the affinity of the

interaction is tighter than 10 pM. So to obtain reliable data from, and kinetic information about, this system requires excellent experimental technique and tests the limits of these instruments' capabilities.

To fully examine this interaction (and to demonstrate the parameters they obtained were not influenced by the assay orientation or immobilization method, as well as to deal with the fact that they could not find regeneration conditions that disrupted the Ab/Ag complex without inactivating the Ab), Abdiche *et al*. used several assay formats, as illustrated in Figure 7A. They tried direct immobilization of both the NGF homodimer and tanezumab Fab, as well as capturing (either by streptavidin or an antibody) of NGF, the mAb and the Fab. In each experiment they worked at very low ligand densities to minimize avidity.

Figure 6. Kinetic analyses of fibre receptor CD46 binding to (A) nine adenovirus fibre knob mutant particles and (B) three knob mutants incorporated into virions. Replicate responses of each analyte concentration are overlaid with the fit of a 1:1 interaction model (red lines). Adapted from Reference 1174 with permission from the American Society of Microbiology © 2008.

Figure 7B shows data obtained from the three biosensors for Fab binding to streptavidin-captured biotinylated NGF. The responses obtained from each instrument are easily discernable above background, concentration-dependent and reproducible. In addition, the Fab could be injected long enough to detect curvature in the association phase. Replicates overlay, indicating each instrument is stable enough to collect data even for the more than 8 h required in each binding cycle. To monitor such a slowly dissociating complex, Abdiche et al. used the SNL approach to collect the full data set from Biacore 3000; with the other two instruments, they performed one-shot kinetics to collect data for all Fab concentrations simultaneously. Although they collected dissociation-phase data for more than 8 h, the Fab/NGF complex did not dissociate enough in each of these experiments to obtain a reliable k_d. Wisely, Abdiche et al. simply reported the k_d was slower than $2 \times 10^{-6} \, s^{-1}$. Taking the comparative study one step further, Abdiche et al. also determined the affinity of this interaction using KinExA 3000 technology. Comfortingly, they found the binding parameters obtained from the surface-based biosensors agreed well and corresponded with values from the solution-based KinExA analysis. So, here's a big raspberry to those who say surface- and solution-based methods never match: Thpppfbt!

Figure 7C and D shows the responses obtained for the interaction tested in the opposite orientation: NFG in solution tested against surface-tethered Fab or mAb. Because the ligands were captured at such low densities, similar kinetic rate constants were obtained for the NGF homodimer binding to the Fab (Figure 7C, top panel) and mAb (Figure 7C, bottom panel) surfaces. In the stepwise saturation experiment shown in Figure 7D, Abdiche et al. tracked the binding of NGF to amine-coupled Fab surface (top) and then followed how Fab in
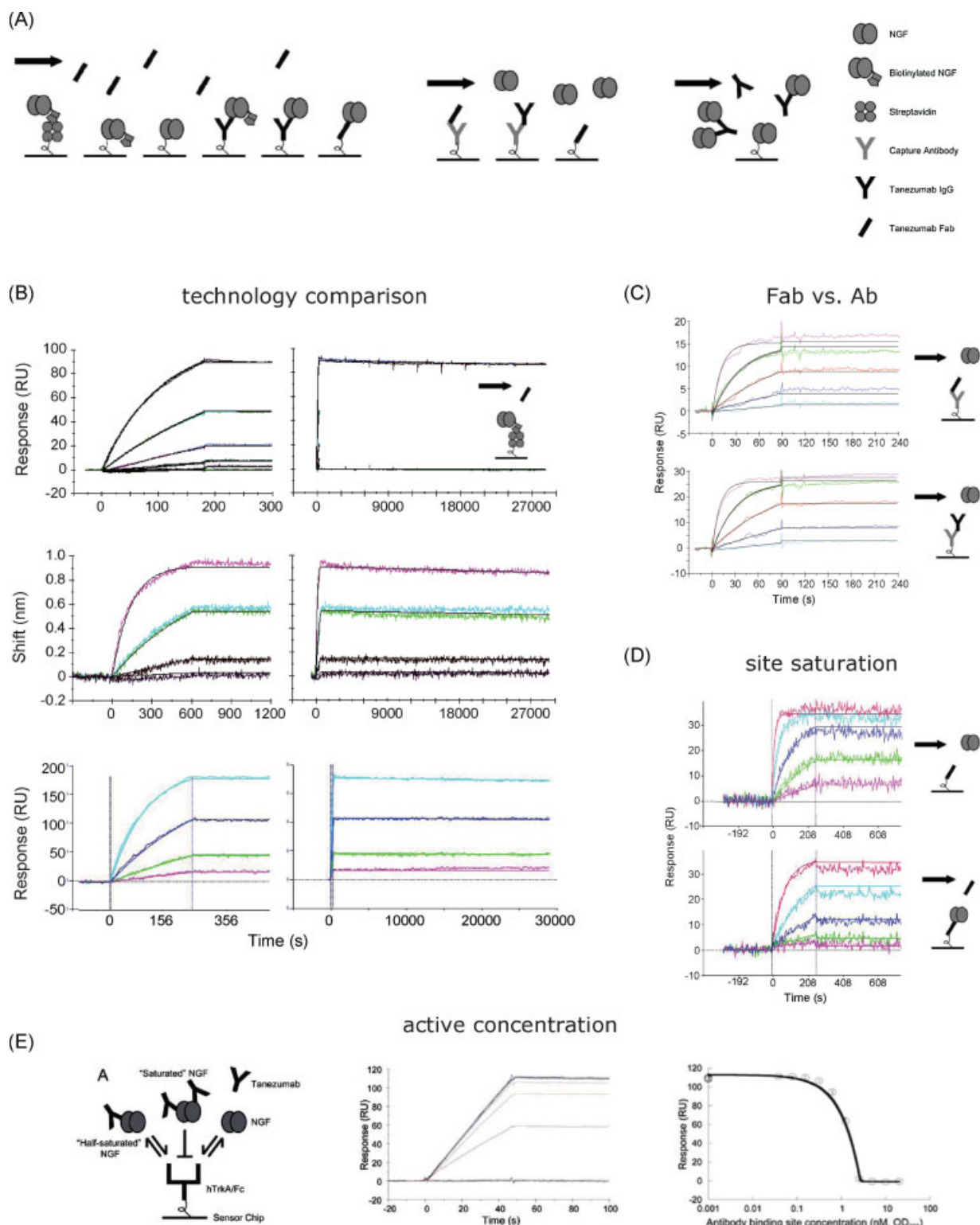
solution bound to the now-formed NGF/FAb complexes on the surface (bottom). Together, the rate constants obtained from the experiments shown in Figure 7B–D confirmed that the kinetics were not influenced by which binding partner was tethered to the surface or by the method used to tether the ligand (either via capture or amine coupling). Thpppfbt, thpppfbt!

And finally, Abdiche et al. developed a biosensor assay to serve as an alternative to functional cell-based assays. Using a solution-competition approach (Figure 7E, left panel), they quantitated the activity of tanezumab in their preparations. The method they described is widely applicable for determining active concentration.

In another comparative study, Abdiche et al. took a slightly different tactic to determine the abilities of the three instruments to characterize a series of peptide/antibody interactions (38). Figure 8A and B depicts the Biacore 3000 'kinetic titration' (also called 'single-cycle') and ProteOn XPR36 'one-shot' approaches for measuring kinetics of peptide binding to immobilized IgG without regenerating between analyte injections. They then compared these results with those obtained from the reverse orientation: Fab in solution tested directly against immobilized peptides (Figure 8C) or in a solution-competition format (Figure 8D). The agreement between the rate constants and affinities determined using different assay designs, interaction orientations and instruments established the reliability of binding parameters reported from the Biacore 3000, ProteOn XPR36 and Octet QK. Nice job.
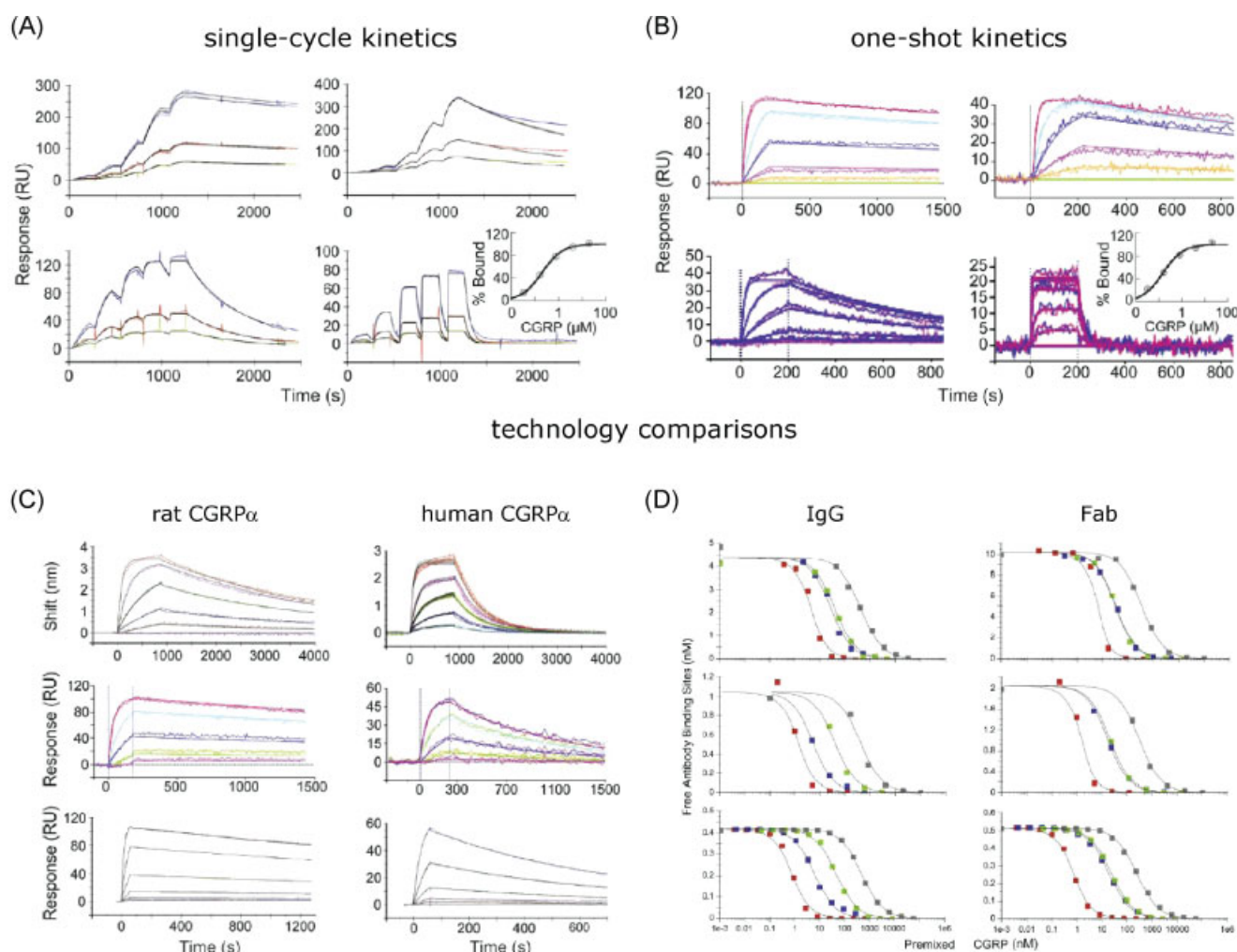
Beusink et al. Angle-scanning SPR imaging for detection of biomolecular interactions on microarrays. *Biosens Bioelectron* **23: 839–844.**

Beusink et al.'s report of IBIS Technologies' iSPR established the capabilities of this array platform (1389). To demonstrate iSPR's

Figure 7. Biosensor analysis of human nerve growth factor (NGF) and its neutralizing antibody, tanezumab. (A) Assay design of the different kinetic experiments performed in this study, with the symbol key at the right. (B) Kinetic analyses of Fab binding to biotinylated NGF captured on surfaces in Biacore 3000 (top), ForteBio Octet QK (middle) and BioRad ProteOn XPR36 (bottom). Left panels show zoomed-in views of the responses obtained during the association phase; right panels are the full sensorgrams, which illustrate the long dissociation-phase data collection times used in these experiments. (C) Kinetic analyses of NGF binding to captured tanezumab Fab (top) and IgG (bottom) performed using Biacore 3000. (D) Kinetic analyses of NGF binding to amine-coupled Fab (top) and Fab binding to Fab-captured NGF (bottom) performed using BioRad ProteOn XPR36. (E) Active-concentration analysis of anti-NGF IgG using Biacore 3000. Left panel: assay design; middle and right panels: responses and inhibition curve. In B–D, the insets depict the assay design for each of these experiments and the black lines are the fit of the replicate responses to a 1:1 interaction model. In the Octet QK experiments, binding was 'sinked' by spiking the dissociation buffer with soluble ligand. Adapted from Reference 39 with permission from The Protein Society © 2008.

Figure 8. Biosensor analysis of calcitonin gene-related peptides (CGRPs) and an anti-CGRP antibody. (A) Single-cycle kinetic analyses of four CGRP/IgG interactions performed using Biacore 3000. Blue, red and green traces depict the responses from high-, medium- and low-density IgG surfaces. In the lower-right panel, the inset shows the binding isotherm obtained from an equilibrium analysis of the data in the main panel. (B) One-shot kinetic analysis of four CGRP/IgG interactions performed using BioRad ProteOn XPR36, with the binding isotherm of an equilibrium analysis shown in the lower-right panel inset. (C) Kinetic analyses of Fab binding to rat (left) and human (right) CGRPα as measured using ForteBio Octet QK (top), BioRad ProteOn XPR36 (middle) and Biacore 3000 (bottom). (D) Solution-based affinity determination of IgG (left) and Fab (right) binding to four CGRPs as measured using ForteBio Octet QK (top), BioRad ProteOn XPR36 (middle) and Biacore 3000 (bottom). In A–C, smooth lines depict the fit of the responses to a 1:1 interaction model. In all direct binding experiments using Octet QK, binding was sinked by spiking the dissociation buffer with soluble ligand. Adapted from Reference (38) with permission from Elsevier, Inc. © 2008.

ability to simultaneously monitor interactions of ligands having widely different masses, these researchers flowed a fluorescently labelled biotin antibody through a flow cell containing spots of biotinylated peptide (2.4 kDa) and biotinylated IgG (150 kDa).

Figure 9A shows how well the binding responses correlated with both analyte concentration and spot density. In the first analyte injection (far left), the signal intensities correspond well to spotted peptide densities (with no signal observed for the negative-control spots). In addition, the responses decrease from left to right as the concentration of injected analyte decreases. From these data, Beusink *et al.* resolved the instrument's limit of detection with respect to the concentrations of both analyte and ligand spotting solutions for this interaction (Figure 9B).
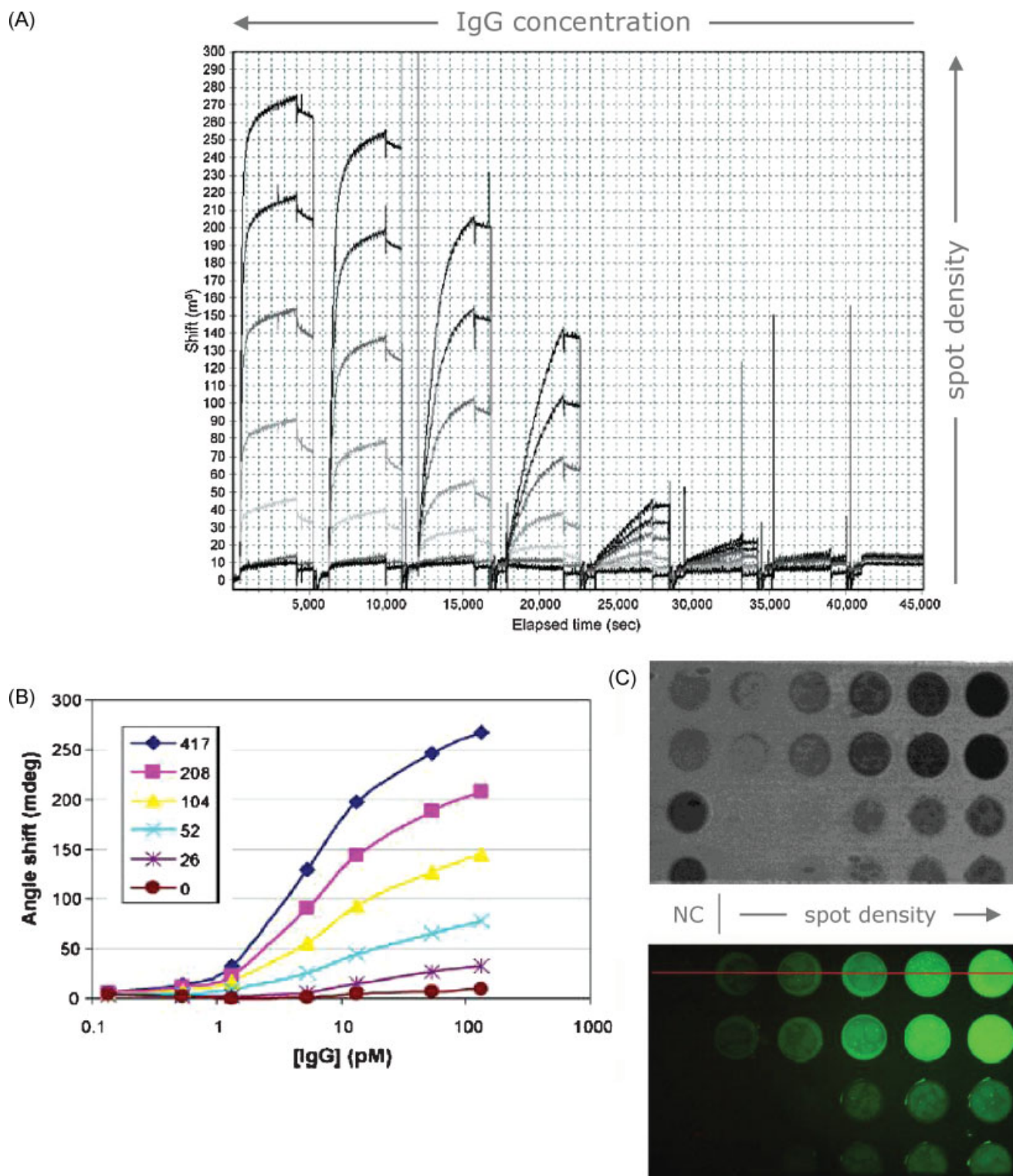
As shown in the top panel of Figure 9C, the SPR image of the chip after the analyte injection confirms these binding results.

Antibody binding to both ligands (two rows each of biotinylated peptide and IgG) is apparent, indicating an array of ligands of different masses could be prepared and characterized. In addition, the intensities of the spots increase with increasing spot density. The correlation between spot density and fluorescence intensity (Figure 9C, lower panel) supports the SPR results. Indeed, this imaging SPR instrument appears promising as one approach to increasing throughput in biosensor experiments.
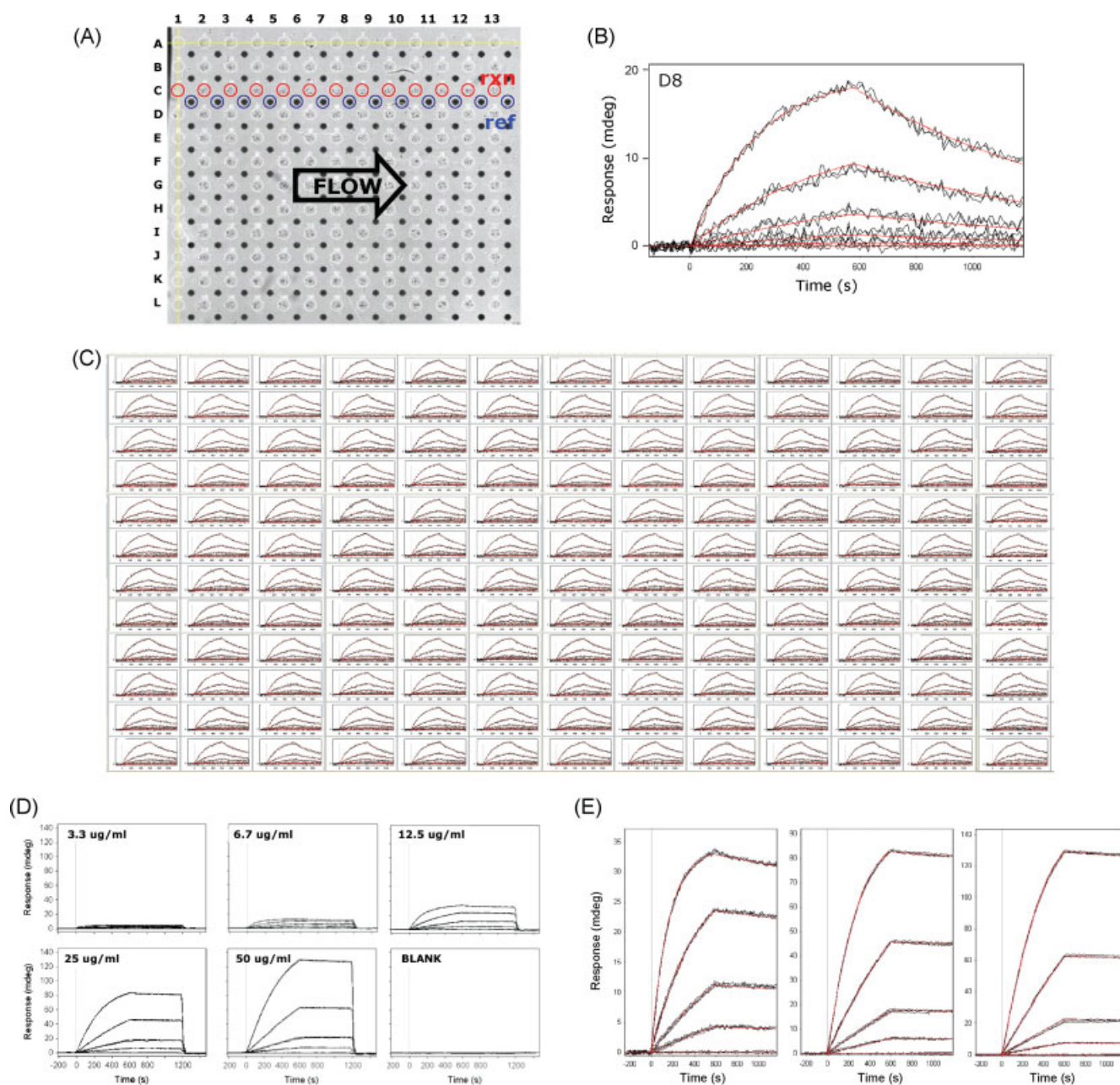
Rich *et al*. Extracting kinetic rate constants from surface plasmon resonance array systems. *Anal Biochem* **373: 112–120**.

Yes, we recommend you to read our paper about extracting kinetics from Biacore's Flexchip system since it demonstrates the potential of array technologies to produce reliable rate constants at spots throughout the flow cell (1362). Figure 10A shows an example of reaction spots within the Flexchip flow cell:

Figure 9. Binding studies performed using iSPR-IBIS. (A) Responses obtained for seven concentrations of an anti-biotin IgG (and a blank buffer injection) binding to five different density spots of biotinylated peptide, a negative-control spot and a background spot. Responses from the first injection (at far left) are for the highest IgG concentration across the seven spots; responses at the far right are from the blank buffer injection across these same spots. (B) Response intensities from panel A plotted against IgG concentration for the five different ligand spot densities. (C) SPR (top) and fluorescence microscopy (bottom) images of biotinylated ligand spots bound with fluorescently labelled anti-biotin IgG. In each image, the top two rows are a peptide dilution series spotted in duplicate; the bottom two rows are an IgG dilution series in duplicate. NC = negative controls (non-biotinylated peptide and IgG). Adapted from Reference 1389 with permission from Elsevier © 2008.

Figure 10. Kinetic analysis using Biacore Flexchip. (A) Screen shot of a 12 × 13 matrix of ligand spots in this large-format flow cell. Reaction regions of interest (ROIs) are circled in white, with one row highlighted in red. The darker circles represent reference spots centred between the reaction spots, with one row highlighted in blue. (B) Duplicate responses (obtained at matrix position D8 in panel A) for a concentration series of rat IgG binding to Protein A/G, overlaid with the fit of a 1:1 interaction model. (C) Responses and fits for rat IgG binding to the uniform-density 12 × 13 Protein A/G spot matrix. (D) Triplicate responses for a concentration series of human IgG binding to spots of different Protein A/G surface density. (E) Global fits to a 1:1 interaction model of triplicate responses for the human IgG concentration series binding to low-, medium- and high-density Protein A/G spots. Adapted from Reference 1362 with permission from Elsevier © 2008.

throughout this 12 × 13 matrix of ligand (Protein A/G) spots, interstitial unmodified regions were used for referencing. Responses for rat IgG binding to one of these ligand spots were concentration-dependent, reproducible and fit to a 1:1 interaction model (Figure 10B). Similar responses were obtained for the entire array of spots (Figure 10C). Across the surface, variability in the rate constants was random and less than 9%. Taking these tests one step further, we tested human IgG binding to a gradient of Protein A/G spots. As expected, binding response

intensities correlated with the concentrations of the ligand spotting solutions and, therefore, spot densities (Figure 10D). These data indicated the Flexchip's lower limit of detection for this interaction was about 2 ug/ml in ligand spotting concentration and about 1 nM in analyte concentration. And finally, Figure 10E shows the global fit of human IgG responses to three of these different density Protein A/G spots. Combined, these studies helped to establish array biosensors as viable platforms for kinetic analyses.

REPORT CARDS

While there were more than 1400 biosensor articles published in 2008, unfortunately the number of really good papers was rather small. It's clear that when you compare the papers *en masse*, too many users aren't putting enough effort into assay optimization, data interpretation and data presentation.
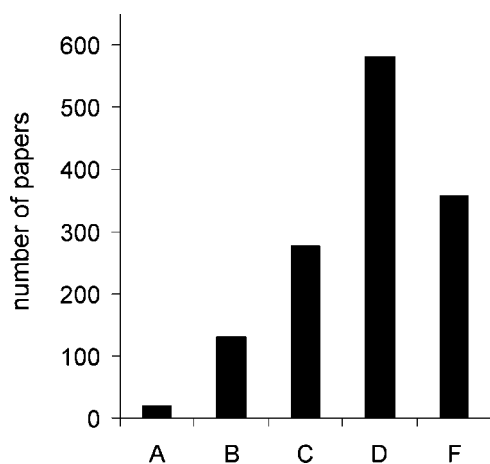
For years, we have discussed how to avoid common experimental errors and stressed the importance of showing data. In fact, in past surveys we used examples from some of the very worst articles to illustrate what is bad with the literature (18). But being the playground bully wasn't really fair since most authors got to stand around and watch us beat up on an unlucky few.

This year we let no one escape. We got out our red pens and assigned each paper a grade. (Now you might ask: "Who put us in charge?" Well, if you want to spend six months of the year searching internet databases, downloading >4000 papers just to find ones that actually describe biosensor experiments, and being exposed to a lot of really bad data, then next year we can send you a pen.) Our grading criteria were based on what would be expected in a high-school chemistry class. To get a good grade back in those days, we were required to do the experiment right, analyse it properly, and present it clearly.

Authors whose papers got *A*'s and *B*'s did the experiment right. Basically, you got an *A* if you showed replicates of each analyte injection and included the fit of a 1:1 interaction model; a *B* usually just lacked the fit or the replicates. *C*'s showed potentially meaningful data that are inadequately optimized: either the experiment or analysis needed more work. *D*'s suffered from a variety of problems. The majority of *F*'s didn't show any data, although a few data-containing papers also failed because their sensorgrams couldn't possibly describe a real binding event.

Now keep in mind that we were only judging the quality of the biosensor data and its presentation, not the scientific impact of the experiments. These grades are to educate users about what they are doing right and wrong, as well as to help readers identify which articles (from a biosensor standpoint) are worth checking out from the library.

The bar graph in Figure 11 shows our grade distribution. Unfortunately, the majority (∼67%) received *D*'s and *F*'s. If you think we were too harsh, just look through the examples in

Figures 12 through 17 and see for yourself the differences in the quality of the work we assigned each grade.

A papers exemplify well-performed experiments and well-presented results. Like all of the examples shown in Figures 12 and 13, *A*-quality responses are single exponentials (indicating they most likely describe actual binding events rather than depict artefacts) and are of what we call "Goldilock's" intensity: not too big and not too small, but just right. Also, the authors showed replicates of analyte concentrations in at least one data set to demonstrate their results are reproducible. It's frightening how few users demonstrate their data are reproducible.

For quantitative analyses, the analyte concentration series spanned a wisely selected range and both data and fits were shown. As illustrated in Figure 12, *A*-quality kinetic papers include the overlay of a 1:1 interaction model.
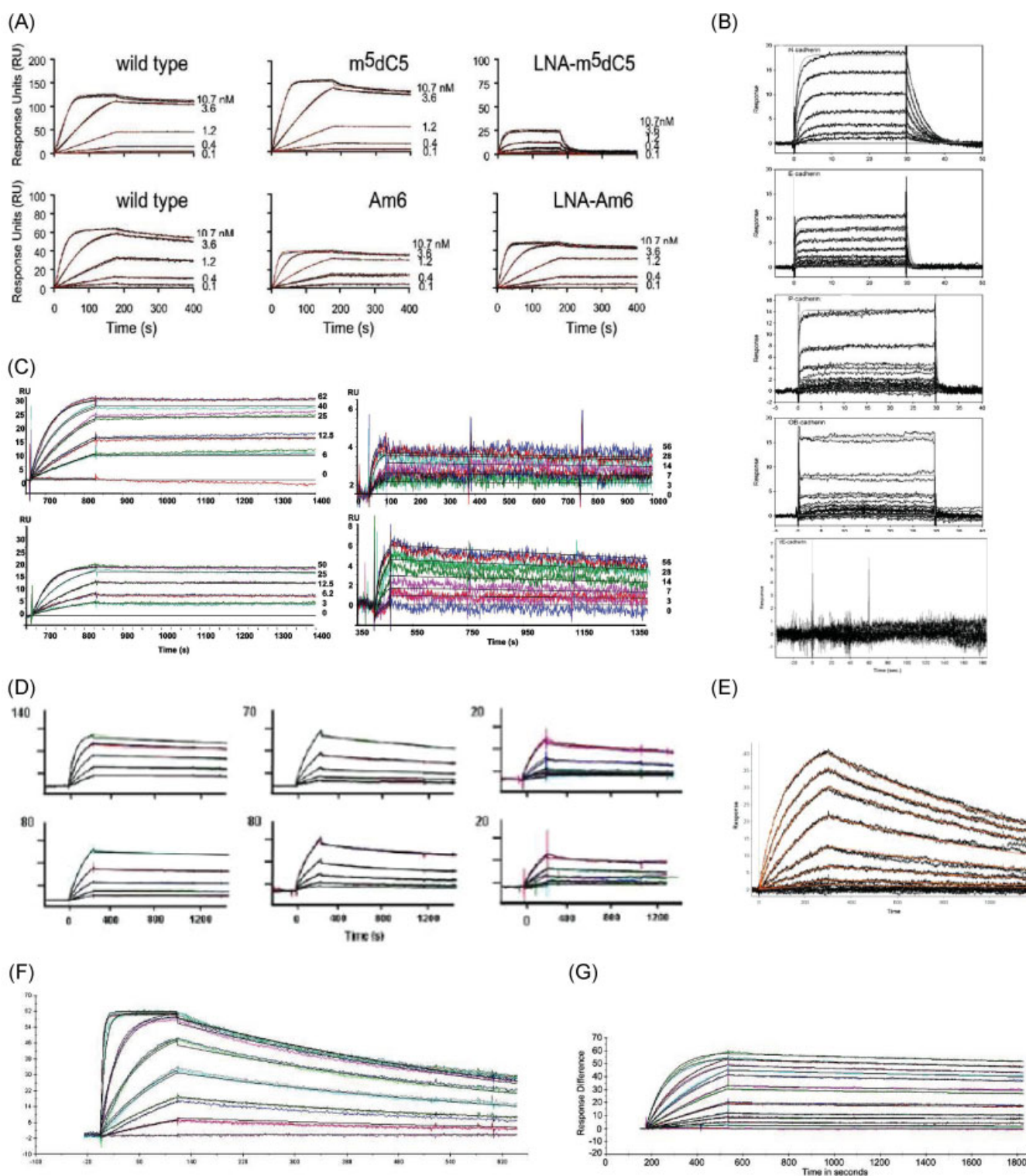
A-quality equilibrium papers (Figure 13) show data sets in which every response reached equilibrium during the association phase and the fit to a simple binding isotherm is included. In addition, each data set in Figure 13 gets a gold star for plotting the binding isotherms with concentration on a log scale. With a log scale, we can clearly see all the data points (compared to a linear scale, which bunches the low-concentration data points all together, making it difficult to really judge the quality of the fit). And note that none of these papers showed a Scatchard analysis. Scatchard plots are so old school we call them 'ancient school'. Using a Scatchard plot was a great idea back in the days when we used an abacus to fit our data. More than likely you have a computer, so just do nonlinear least-squares fitting. (Are we going too fast for you?)

B papers typically lack only one of the features we look for in a biosensor analysis. It's frustrating because sometimes the authors show replicates which are beautifully overlaid but then fail to show a fit to the data (Figure 14A). Most often, they show a great fit to a simple model and are only missing replicates (Figure 14B–H). Clearly, these authors know what they are doing—with just a little more work they would get an *A*. And finally, the variety of experiments (e.g. screening, standard kinetics and equilibrium analyses, kinetic titrations and stability tracking) that produce at least *B*-quality data demonstrate that every assay format can produce data at least as good as these.

C papers present data that are most likely due to an actual binding event, but the experiment and/or analysis needed to be improved before publication. For example, Figure 15A shows what we call a 'pearls-on-a-string' analyses. These data sets have a very narrow dilution series. We love that the authors ran a lot of analyte binding tests, but the data sets would be much more informative if there were replicates of fewer analyte concentrations instead of singlets of such a narrow dilution series. Andy Warhol said 'Replication is the key to success, now who wants soup?'

Figure 15B shows two examples of complex responses that are difficult to attribute to a specific binding event. Most likely, one (or both) of the binding partners is poorly behaved and the experimental conditions could be better optimized. As we've said many times before, high ligand densities and high analyte concentrations lead to complex binding. You need to realize that there is a limit to the highest concentration you can go to with many proteins and small molecules before you introduce complicating factors like non-specific binding, crowding or aggregation. Less is more, more or less.

Figure 15C shows two data sets that were fit to a drifting-baseline model. Unfortunately, this model may not really
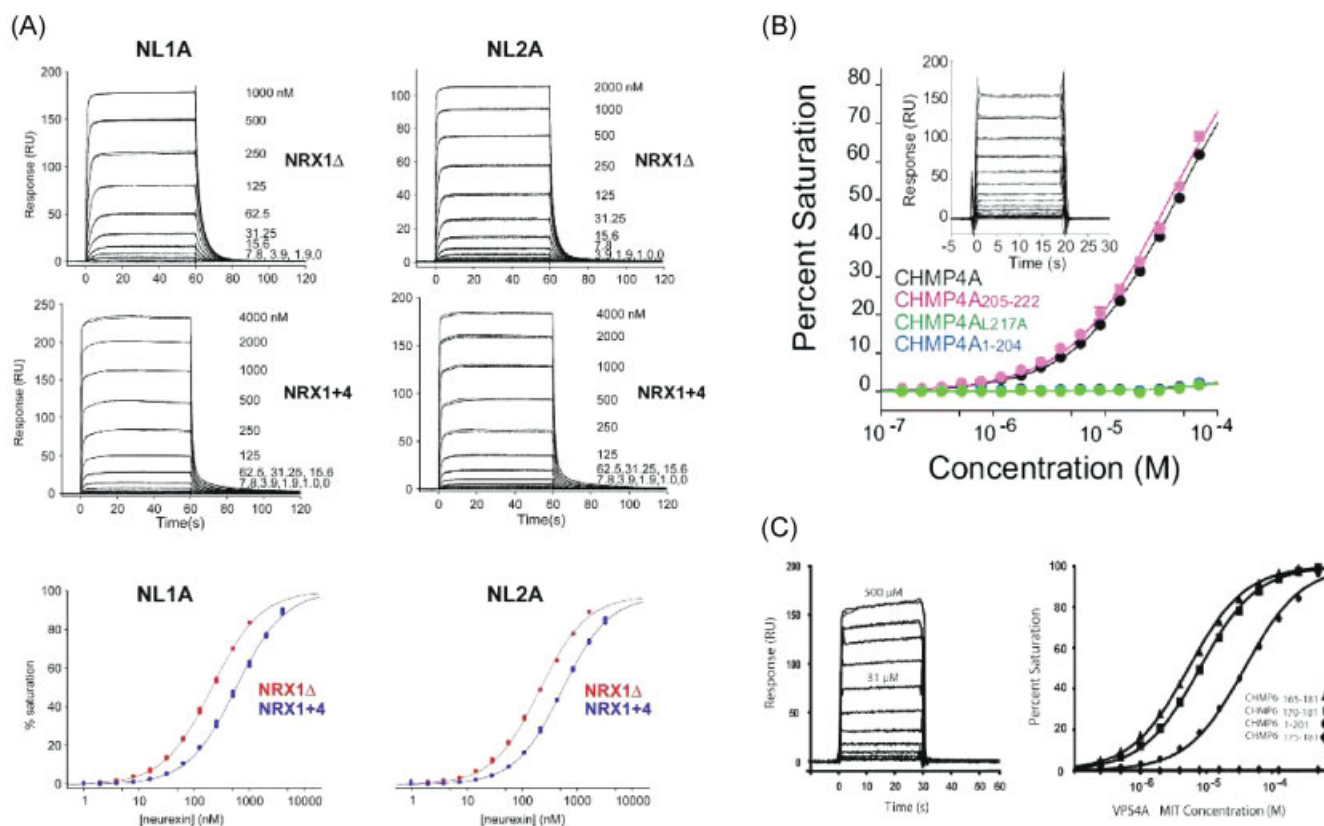
Figure 11. Bar graph of the grade distribution for the 2008 literature.

Figure 12. Examples of *A*-quality kinetic analyses. Adapted from References (101,282,449,452,576,709,846) with necessary permissions granted by Cold Spring Harbor Press, the American Chemical Society, Elsevier, Inc. and Springer © 2008.

account for what's going on in the reaction. It's just a crutch that many groups lean on to get a good fit to poor-quality data. We call this fitting to a 'something–something model' since it is unclear what portion of the response is accounted for by the drifting-baseline model (some sensorgrams drift up and some drift down). It is far better to improve the integrity of the data by

optimizing the experimental system than to model your brains out.

The experiments shown in Figure 15D and E are some of the easiest to improve. To obtain reliable rate constants from a kinetic analysis, you need to have curvature in the association phase and observable decay in the dissociation phase. So to get more

Figure 13. Examples of *A*-quality equilibrium analyses. Adapted from References (178,185,224) with permission from Elsevier, Inc. and the National Academy of Sciences USA © 2008.

curvature in the association phase of the data shown in Figure 15D, the authors just needed to inject the analyte for a longer time or test higher concentrations. (Granted, the injection time in the right panel was more than 40 min but that still wasn't long enough. Alternatively, you might try kinetic titrations if you are limited by injection volume.) And all of the examples in Figure 15D and E need longer dissociation times. Most biosensors are automated, so why not just let it run to collect more data in the dissociation phase? A well-conditioned biosensor should be able to collect reliable dissociation data for 12 h or more if you are careful and patient.

D papers give biosensor technology a bad reputation, but unlike Joan Jett, we do give a damn about a bad reputation. For example, some *D*-quality experiments produced responses that might actually describe binding, but they are so weirdly shaped it's hard to tell what is going on (Figure 16A) or display complex binding at high analyte concentrations (Figure 16B). It's apparent that most users whose papers got *D*'s fail to understand a sensorgram should be a single exponential. (Can you draw what a sensorgram should look like? It's not just some arbitrary curve. It has a specific shape. Knowing how your data should look is Step 1 in improving biosensor analysis. Now you only have 11 steps to go.).
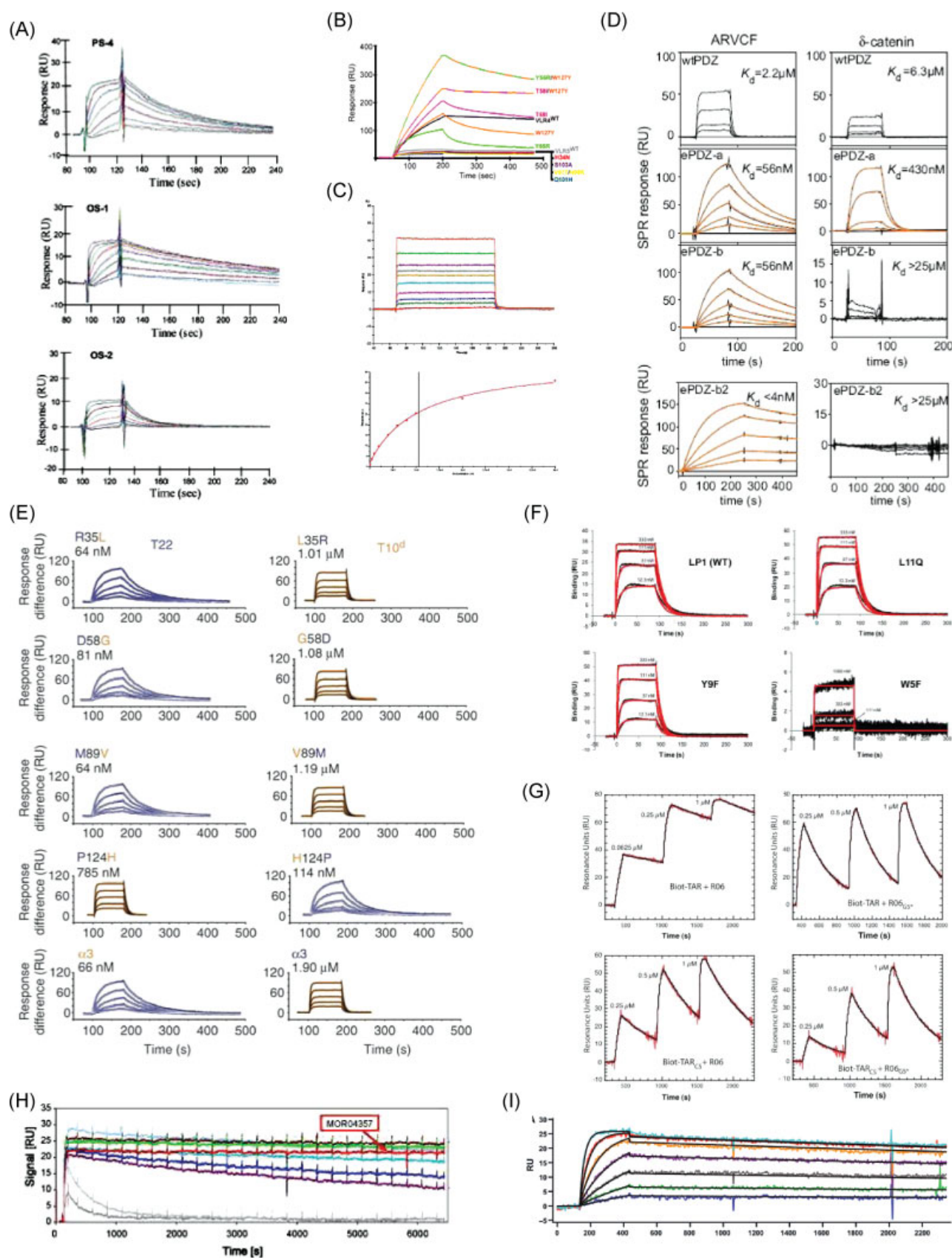
There are two things that made us so mad these papers automatically got *D*'s. If you used an equilibrium analysis to determine equilibrium binding constants from responses that did not come to equilibrium (like those in Figure 16C), you got a *D*. (Actually, you should have gotton an *F* for *False* reporting of binding constants.) It's also common to see people plot equilibrium responses against concentration but not include a fit, as in

Figure 16D. They do this because they either don't understand how to fit the data or they fit it to a simple binding isotherm and it didn't fit well so they choose not to show it. Also, just connecting the data points with a line is not fitting the data.
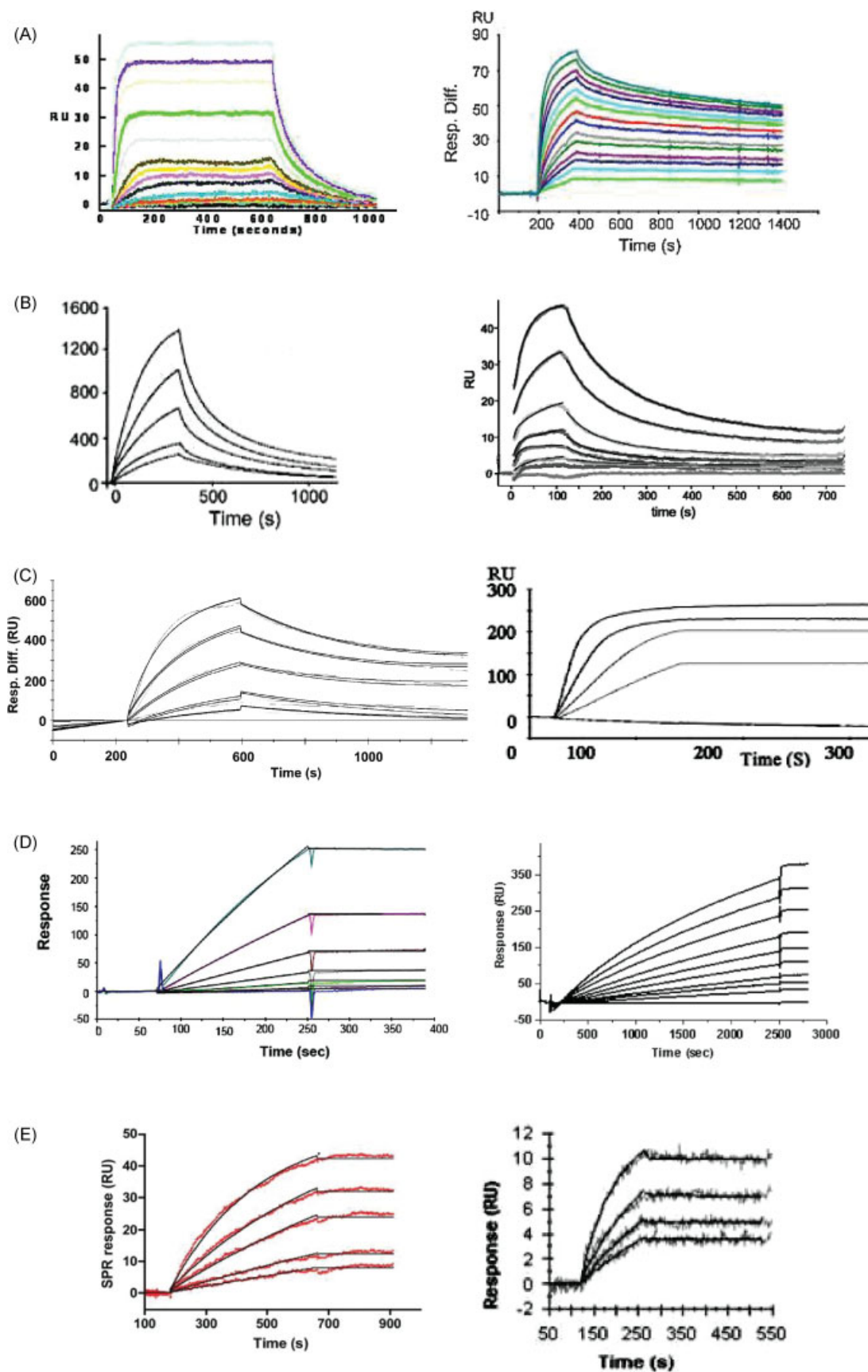
And if you fit your kinetic data using a conformational-change model (e.g. Figure 16E), you also got a *D*. We will talk more about this later but what's funny is that most people who used a conformational-change model said something like 'we used a conformational-change model because it fit the data better than a simple model'. Nice going, Einstein. You do know that you can't prove a model is correct by simply fitting it to the data? (If that sounds new to you, then go ride in an elevator at constant velocity and tell us if you are moving.) What's even funnier is that most users of the conformational-change model don't even report the correct units for the conformational-change step. And the same can be said for those who use a bivalent analyte model. Nothing says 'I don't know what I'm doing' more than not knowing what you're doing.

And another thing about model fitting. We were sad and disappointed to see how many authors went model surfing. They fit their data sets to just about every available model, showed all these fits, and reported the rate constants obtained from each fit. It shouldn't be left up to the reader to figure out what is going on in your reaction. Do they expect us to do their homework for them?

Most F papers show no data whatsoever but still report binding constants (my dog ate my homework). Without seeing the data, we have to wonder if the authors of *F* papers did the experiment right or, in fact, did it at all. With today's opportunities to include data in on-line supplements, the excuse that 'there's no room in
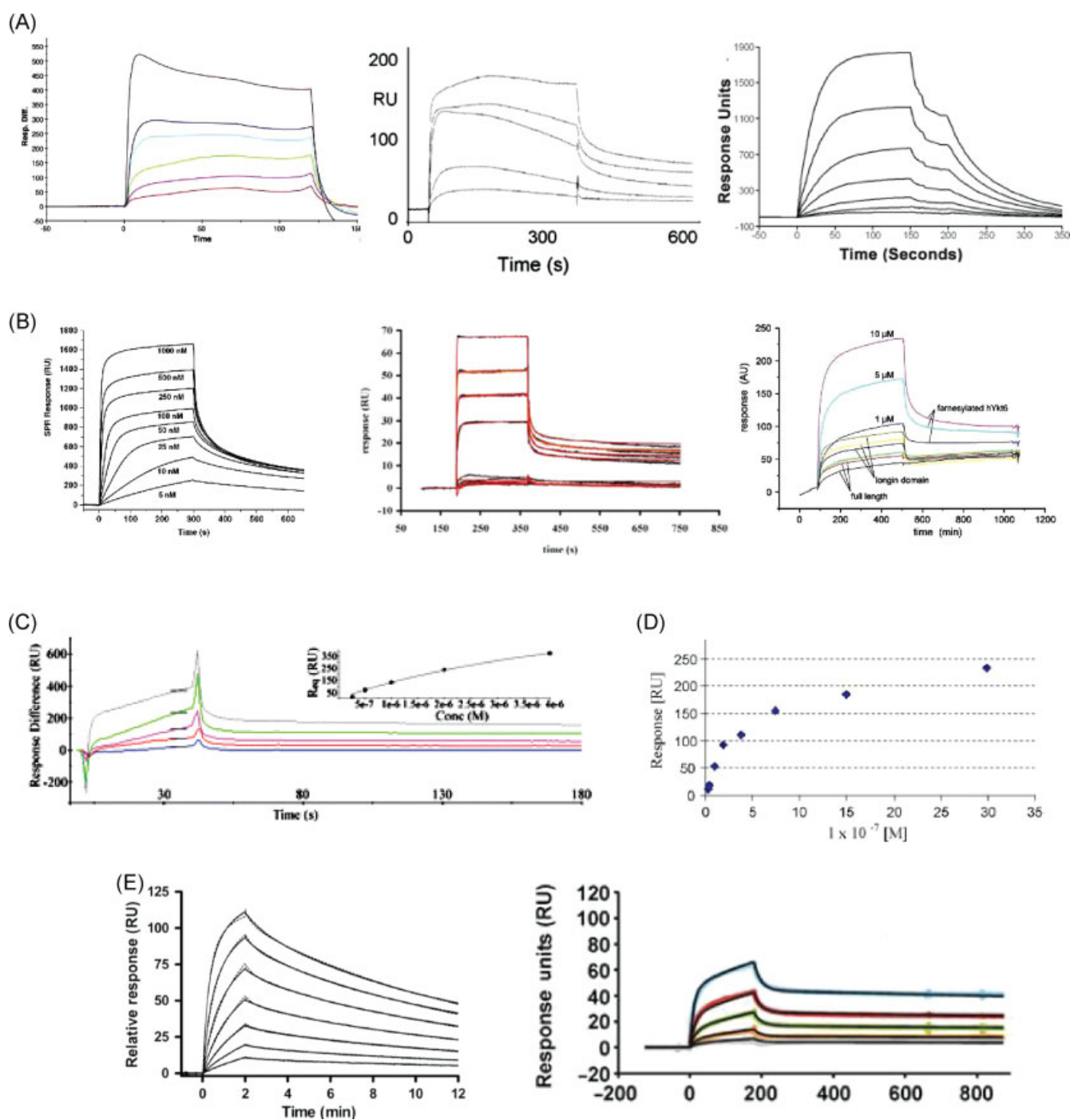
Figure 14. Examples of *B*-quality data. Adapted from References (384,411,470,505,697,728,731,852,997) with necessary permissions granted by Macmillan Publishers Ltd: [Nature Immunology], Elsevier, Inc., the American Chemical Society, the National Academy of Sciences USA and Wiley © 2008.

Figure 15. Examples of *C*-quality data. Adapted from References (93,192,261,377,472,653,686,881,918,1030) with necessary permissions obtained from the American Chemical Society, Elsevier, Inc., Wiley Interscience and BioMed Central Ltd. © 2008.

Figure 16. Examples of *D*-quality data. Adapted from References (216,225,344,523,529,762,819,951,1074,1097) with necessary permissions granted by Elsevier, Inc., the American Chemical Society, the National Academy of Sciences, USA, Macmillan Publishers Ltd: The EMBO Journal and the American Society for Cell Biology © 2008.
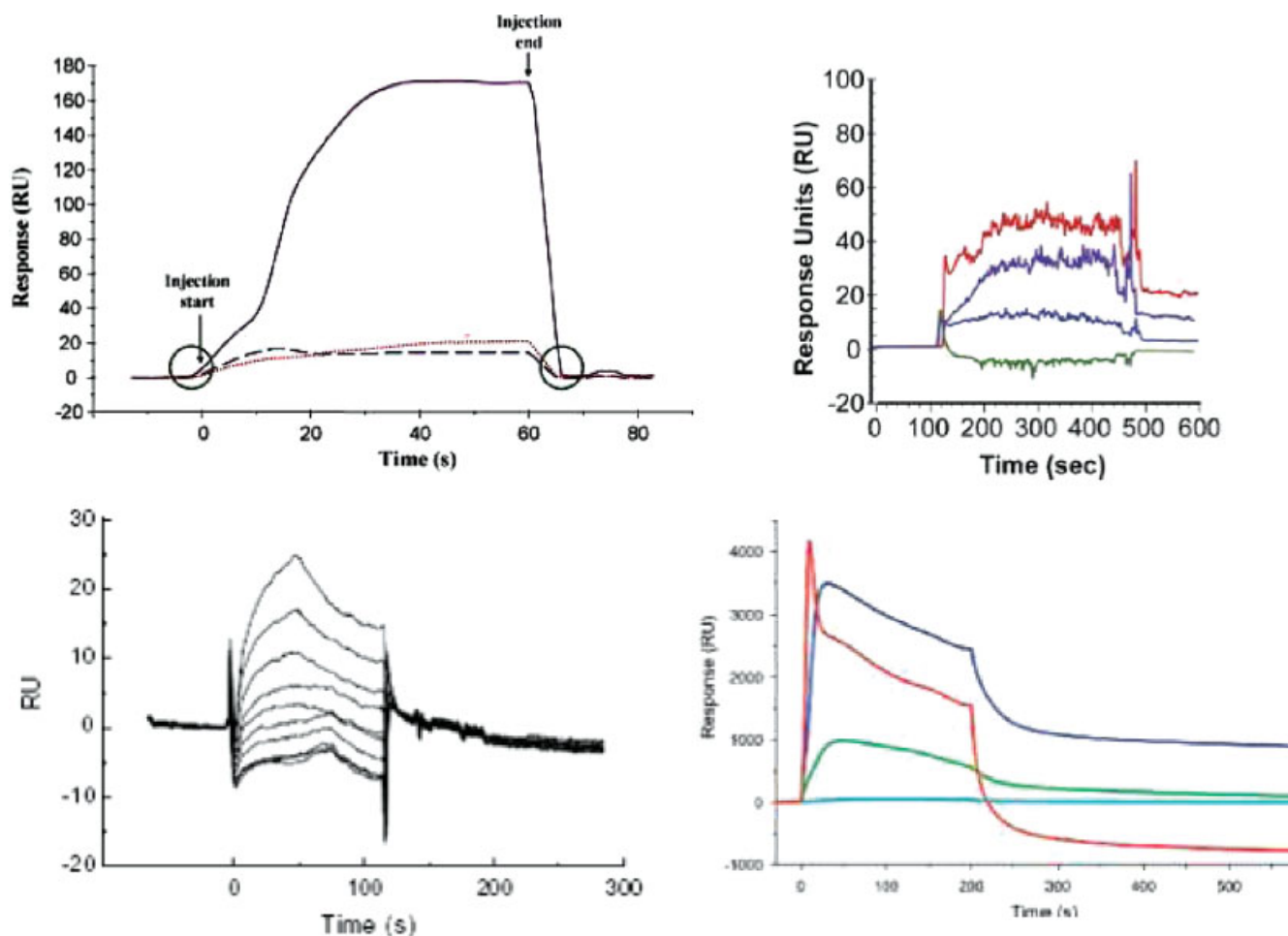
the paper' doesn't fly. It's unforgivable 01to report binding constants without a figure of the data. Come on, it's show-and-tell. You spent all that time collecting data. Why not share it with the rest of the class? We know the answer. It's like Michael Jackson said:

'Because it's Bad, it's Bad—come on
(Bad Bad—really, really Bad)
You know it's Bad, it's Bad—you know it

(Bad Bad—really, really Bad)
You know it's Bad, it's Bad—you know it, you know
(Bad Bad—really, really Bad)
And the whole world has the answer right now
Just to tell you once again... who's Bad'

Who's bad? Your data are bad. Other *F papers* include some data, but they are absurdly awful. Figure 17 shows examples of

Figure 17. Examples of *F*-quality data. Adapted from References (276,916,1075,1134) with permission from Elsevier, Ltd. © 2008.

published data that are in desperate need of improvement. We wish Michael was still around to help us write a catchy song about that.

> *Who can generate poor-quality data?*
> *(we can, and we do)*
> *Who publishes whatever comes out of the machine*
> *Often without even showing the data?*
> *(we can, and we do)*
> *All the time!*

Okay, we will stick to writing literature reviews rather than songs if you promise to put a little more effort into your experiments and publications.

DETENTION

Although the bell may have sounded, school's not over for some of you. Sit back down. We need to talk with you about a few problems that are showing up in the literature with increasing frequency.

Bulk-shift INcorrection

The bulk refractive index correction was introduced to account for small residual jumps in signal that may be apparent when working with low-intensity responses. The correction involves
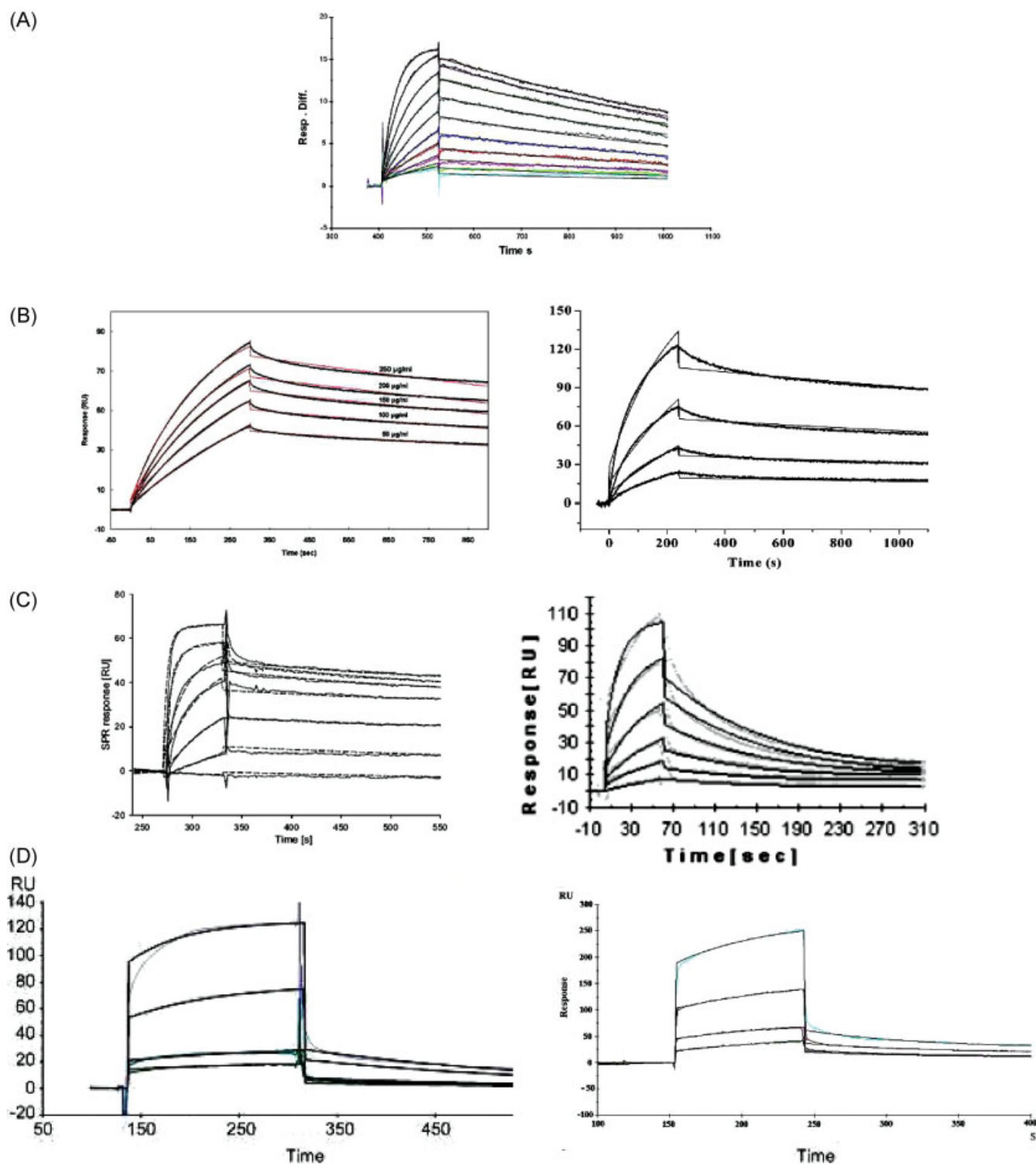
modelling a step function of equal intensity throughout the association phase. Figure 18A is a good example of a data set that contains this small jump in the association phase and Mattu *et al.* were justified in including the bulk-shift correction when fitting their data. (Excellent fit to the data. This paper would have received an *A* if this group had run some replicates and not so many pearls on a string.)

In contrast, Figure 18B–D shows several data sets in which the bulk-shift correction should not have been used. We call these data sets 'bulk-shift INcorrection' because this correction factor was used to mask complexity in the responses. Notice how the bulk shift improves the fit but it is not really describing the complex kinetics in the data set itself. You often find users like these stating 'the data fit well to a simple 1:1 interaction model' when in reality they are not using a simple model at all. The two examples in Figure 18D take this to an extreme level and use the bulk shift to account for over 80% of their binding signal. Don't let these groups fool you.

Do you see what I see?

People often ask us why we insist on seeing fits to the data. A fit instantly shows the reader you know what you were doing (and showing the data overlaid with the fit does not take up any more space in the article so we don't want to hear any excuses).

To illustrate the importance of showing fits to your data, we simulated what the fit would have looked like in several data sets
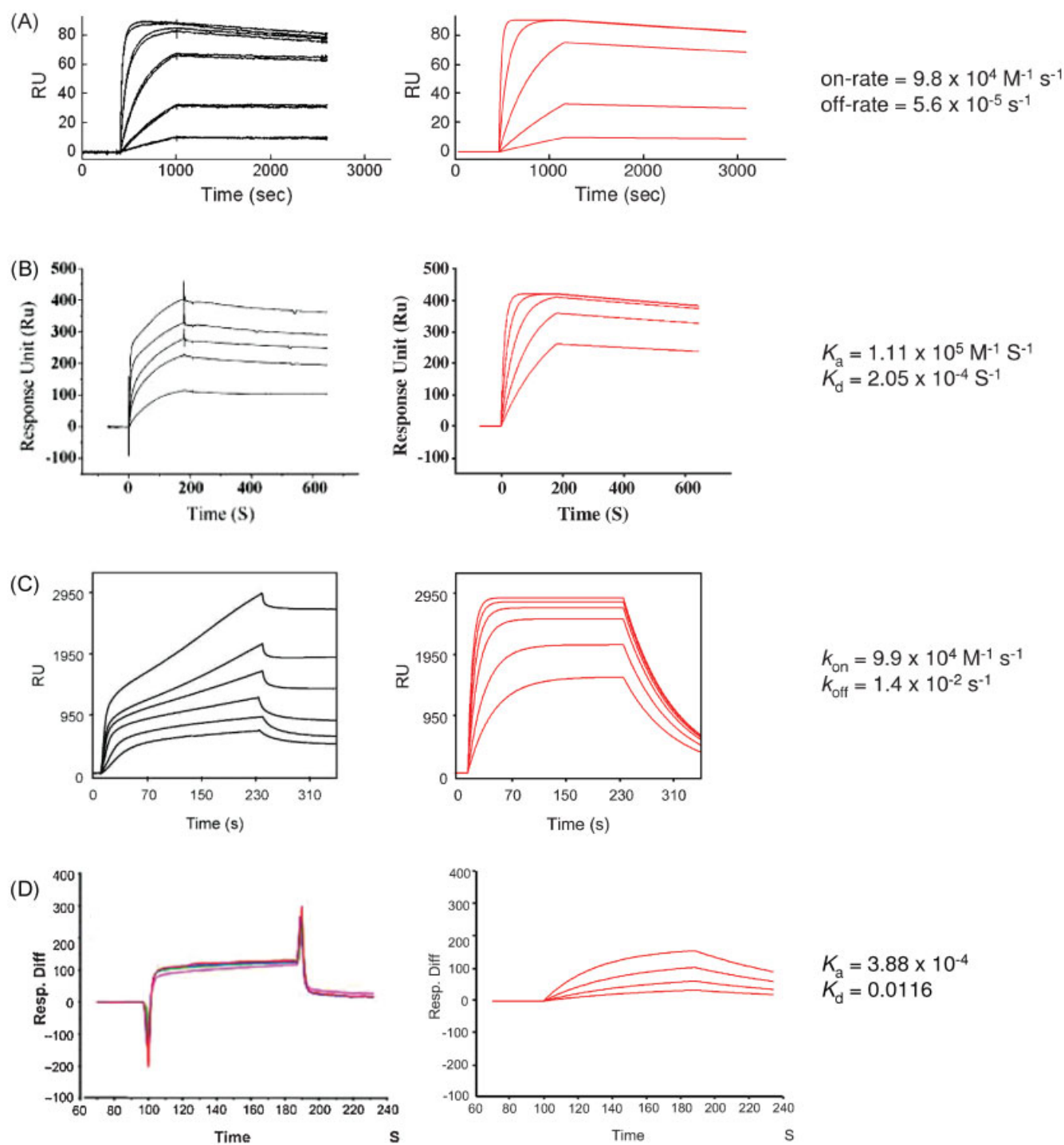
Figure 18. Examples of using the bulk-shift correction (A) appropriately and (B–D) inappropriately. Adapted from References (136,403,778,746,839,947,1055) with permission from Elsevier, Inc., Springer and the American Chemical Society © 2008.

(shown in Figure 19). Remember, each of these papers showed a figure of data (shown in the left panels of Figure 19) and included rate constants (listed on the right) but did not include the kinetic model fit overlaid with the data. Our simulated responses are shown in red.

In Figure 19A, our simulation closely mimics the authors' data, indicating their reported rate constants are believable. This data

set even included replicates; if these authors had just shown the fit, their paper would have received an A.

The data in Figure 19B–D are a different matter altogether. In most cases, the authors' data do not look anything like our simulations, leaving us to wonder where they got their binding parameters from. We call these 'hate constants' because we hate to see numbers published that are so misleading.

Figure 19. Data sets (left panels) for which we simulated responses (right panels) based on the rate constants reported by the original authors. Adapted from References (198,220,355,651) with permission from Elsevier, Inc. and Informa UK Ltd. © 2008.

Even by just glancing at the data in Figure 19B–D, you should be able to spot some problems. For example, you should know that a k_d of $10^{-2}\,s^{-1}$ is relatively fast, unlike the authors' responses shown Figure 19C. We don't think you should be allowed to step up to the biosensor unless you can do a few simple things, like recognize some ballpark rate constants (at the very least, you should know what the profiles of different off rates look like). Then you can tell if the parameters you report are at all close to what you'd expect.

And then there is the data in Figure 19D. Fortunately, we weren't the only ones who were surprised by the rate constants reported from this data set. In their Letter to the Editor, Dolimbek *et al.* commented (8), 'The Biacore experiments (in Reference 651) don't make a lot of sense. The methods do not seem to fit the experimental data. There is no dose-dependent response. . . . . . .a much greater response differential would be expected. But the results are not what would be expected, and it is uncertain what they were actually flowing over the cell. Hence it is questionable

how the affinities were calculated'. It's so gratifying to see members of the community taking a more active role in teaching one another (maybe next year we should send Dolimbek a red pen). We also want to point out that the authors of Figure 19D reported an association rate constant of 3.88×10^{-4}. Really, you have an association rate constant that is 10^{-4}? That seems a tad slow to us.

Cha-cha-cha-changes

Why do people persist in clinging to a conformational change model simply because it fits their data? As we pointed out earlier, you cannot prove a model is correct by fitting it to a data set (jump on that elevator). The reason is that multiple models may fit the data equally well. Of course, the only way to prove a model is correct is through experimentation. To prove the conformation change model is applicable to your interaction, you need to test analyte binding for different lengths of contact time. Most users forget, however, the key is that *you have to saturate the surface for each contact time*. Otherwise the method cannot be used to distinguish between a conformational change and something else like surface heterogeneity.

Murat *et al.* demonstrate the correct way of testing for a conformation change (Figure 20A). This group collected binding data with different association times, but notice how the response for even the shortest contact time reaches the same magnitude as that of the longest contact time. And notice that the units for the response are still in RU, which is correct (they didn't rescale or normalize). They appropriately showed that contact time did not influence the dissociation phase in their system. They get to go home early today.
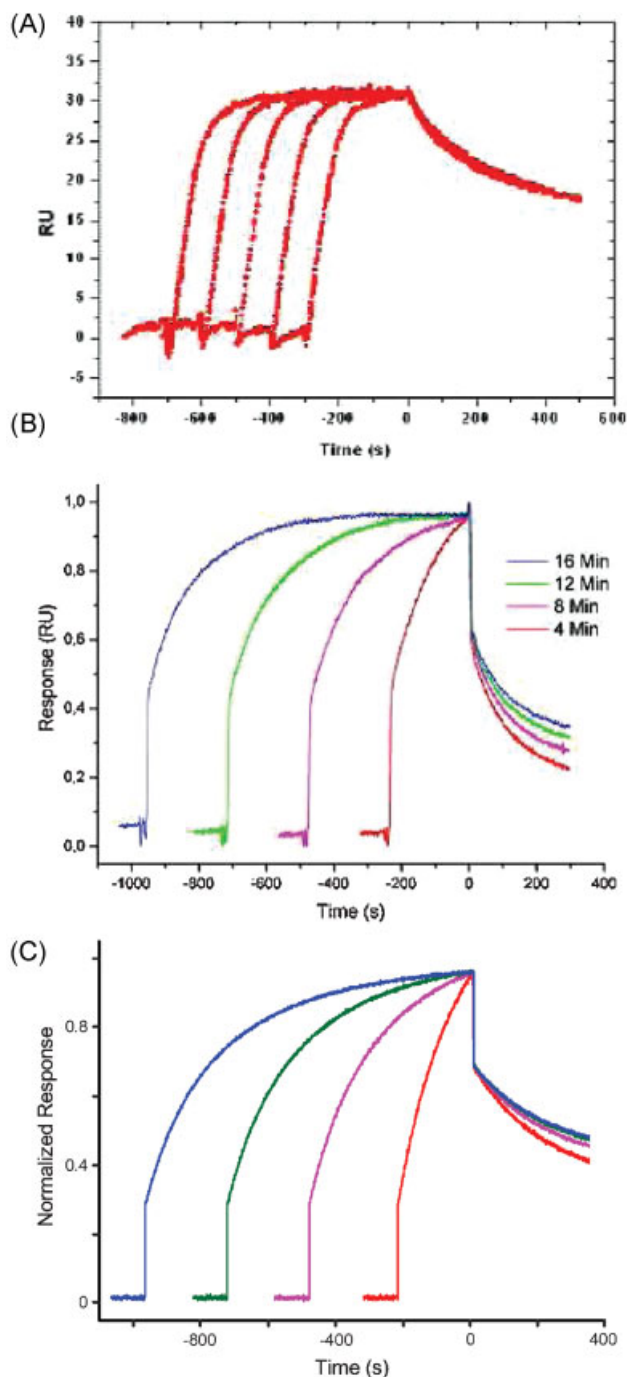
The data in Figure 20B show how NOT to test a conformational-change model. Sure, these authors varied the association phase time and saw differences in the dissociation rates, but look carefully at the units of the *Y*-axis. They scaled the responses at the end of the association phase but tried to hide this rescaling by naming the *Y*-axis 'Response (RU)' when it's clearly not. It should be labelled 'Normalized Response' and it would not have units. It's also clear to see, in the shapes of the response profiles collected for different times, that the reactions have not all saturated the surface, which is required for this test. So while the dissociation phases for the different contact times of analyte look different, this does not prove it's a result of a conformational change.

How can we be so sure? Take a look at the responses in Figure 20C. These look strikingly similar to the ones in Figure 20B and they also clearly show differences in dissociation rate that correlate with contact time. Raise your hand if you think this proves that this data set is a result of a conformation change reaction. You can put your hands down now. You're wrong. We simulated this data set with a surface heterogeneity model.

We could have simulated similar looking data using a solution heterogeneity, a bivalent analyte and/or a aggregation model. Don't be fooled by people claiming to prove they have a conformational change when in fact they don't know what they are doing. And how relevant do you think a so-called conformational change is when it occurs on a time scale of 10–15 min? You know Occum's Razor: 'the simplest model is correct'. Well we have a new razor for you, Myszka's Razor: 'Stop using a conformational change model to fit your poor-quality data'.

FINISHING SCHOOL

In order to standardize the biosensor literature, consider these recommendations as your new school uniform.

Figure 20. Data sets in which the injection time was varied. Panels A and B were adapted from References (798,953) with permission from Elsevier, Inc. and Wiley-VCH Verlag GmbH & Co. © 2008.

What's in a name?

My mother [DGM] always said 'If you can't say anything nice, don't say anything at all. And never drink wine out of a bottle bigger than your head'. Well, we've got something to say to those of you who either don't proofread your publications or don't know how to report binding constants in a paper.

Here's the deal. The IUPAC IUBMB, which is the international governing body responsible for standardizing scientific language, defines the association event for a biomolecular interaction as k_a

and the dissociation event as k_d. Note it's a small letter k that is in italics and a subscript little a or d that is not in italics. Period. They are not K_a, K_A, k_{for}, k_{+1}, k^a, k^{ass}, k_a, k_{assoc}, k_{on}, $k_{a\,app}$ and K_d, K_D, k_{rev}, k^d, k_d, k^{diss}, k_{dissoc}, k_{-1}, k_{off}, $k_{d\,app}$. It is truly amazing the variety of notation you can find in the literature. If you don't believe us, look back at the binding constants reported in Figure 19. These were taken directly from the authors papers without editing. Notice that none of these four examples conform to the IUPAC standard for reporting rate constants. And the same can be said for the equilibrium dissociation constant, which should be presented as K_D. Never report your affinities as an equilibrium association constant (K_A); you're not doing isothermal titration calorimetry here (yes, yes we know $K_D = 1/K_A$ and so does everyone else so you don't need to provide both in your paper).

Reporting the correct units for parameters is also important. The standard units for the association rate constant are $M^{-1}\,s^{-1}$ and the dissociation rate constant is s^{-1}. We've seen the association rate constant reported with units of $M\,s$, M/s, s/M, $mole^{-1}\,s^{-1}$, M and just s. About 15% of the time there are no units at all. It may seem like nitpicking, but is it really that hard to report binding constants with the correct units? Plus it gives the reader of your paper a sense that you know what you are doing.
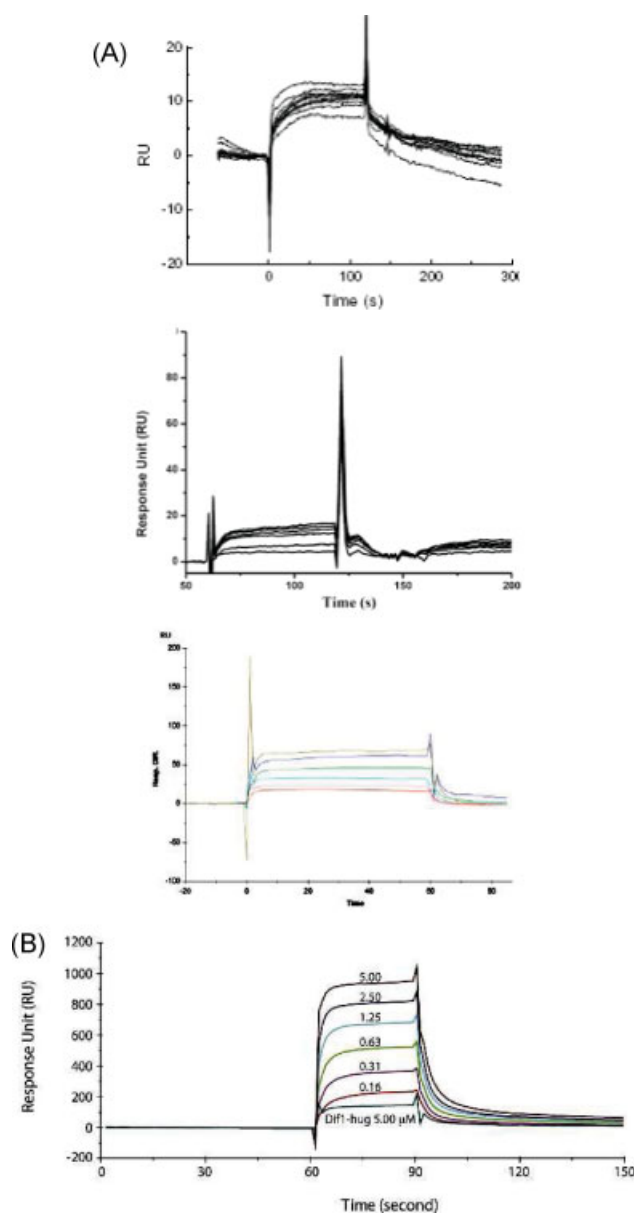
Show me the error of my ways

Now if you really want to impress your colleagues, you should present the binding constants with the correct standard errors. (Gosh, at this point we would be happy if you reported any errors at all). An association rate constant like $5.25 \times 10^4\,M^{-1}\,s^{-1}$ is actually meaningless without standard errors associated with it.

There are two types of standard errors that we are concerned about, statistical and experimental. Statistical errors are presented by the fitting software, typically to what is referred to as one standard deviation which has a confidence interval of 68% (see Wikipedia for details on confidence intervals; we're limited in space here). Basically, it defines how well we know a parameter in the model for a given data set. Now many users are shocked to see that the statistical error may be really tiny, like only a tenth of a percentage of the parameter value itself. For example, in an information-rich (lots of curvature) data set, the computer may tell you the association rate constant is $(5.2578 \pm 0.0015) \times 10^4$. The fact that the standard error is small compared to the parameter value tells us in this case that the association rate constant is well defined. If the computer said the association rate constant was $(5.2578 \pm 1.5245) \times 10^4$, this would say the parameter is not as well defined. However, it doesn't mean the value is wrong.

Now it is just as important to report the correct number of significant digits when publishing binding constants. You can't simply publish whatever numbers the computer provides you. (Open the pod bay doors, Hal.) The rule is to round up the value of the error to its first significant digit. This then defines the number of significant digits to report in the binding constant. So, the correct way of reporting the first example in the paragraph above would be $(5.258 \pm 0.002) \times 10^4$. You see how we rounded up the value of the error as well as the binding constant to four significant digits? The second example would be reported at $(5 \pm 2) \times 10^4$. Here, you try one. How would you report the dissociation rate constant if the computer gave you $1.8309 \times 10^{-3} \pm 5.7251 \times 10^{-5}$ to you? If you said $(1.83 \pm 0.06) \times 10^{-3}$, you would be correct. If you got it wrong, read this paragraph again.

Of course, statistical errors tell us only how well a parameter is defined for a model within a particular data set. To define the experimental error you would need to repeat the entire study. This may sound crazy but hear us out. To be a scientist means you often have to run an entire experiment more than once to see how unknown variables affect the results. You would then take the average of the measurements for the parameters and use their standard deviation to report the experimental standard error. We could find only a handful of papers which provide convincing evidence that they replicated the entire study. Unfortunately, it seems that fully automated instruments have made us complacent and spawned an attitude of 'If it came out of this fancy and expensive machine with a computer, I don't need to repeat my experiment'. (Hal! Open the pod bay doors!).

Figure 21. Figures scaled to emphasize (A) the spikes and (B) the baseline. Adapted from References (194,916,919,940) with permission from Elsevier, Inc. and the American Chemical Society © 2008.

Tuck in your shirt

For years we have been encouraging users to show figures of their data in publications, and we are happy to see that a majority of papers (>70%) now do present some data. The next issue that we would like to address is just how the data are presented. One of the common and comical ways we see data presented is illustrated in Figure 21A. Note how these figures have been scaled so that the spikes (which often occur at the transitions between running buffer and sample) are visible. This forces the responses (the curvy parts) that we really care about seeing to be really small. We call this kind of plotting 'scaling-to-the-spikes syndrome' or 'SSS plotting' for short. The data shown back in Figure 19D are another good example of this.

Another common ailment in plotting is to show too much baseline before or after the injection. While the binding data presented in Figure 21B are in fact pretty good, it sure would have been nice if the authors focused on the response curves so we could see them better (and the overlay of the fit if they had included one) rather than consuming half the space in the figure with a flat line.

Compare the sloppy plots of Figure 21 with those shown in Figures 12 through 14. You see how easy it is to interpret well-presented data?

Another way we want to further standardize the biosensor community is to encourage everyone to publish good-looking figures. Too often authors simply dump low-resolution screen-shots from the biosensor software directly into their paper. Oftentimes, this means the figure is scaled to include those irrelevant data spikes and/or the lines are too thin or too faint and the axis labels are too small to read. Making a good-quality figure is an essential part of becoming a professional biosensor scientist.

BIOSENSOR PLEDGE

Now before we finish with today's exercises, we have homework for you. Cut out the Biosensor Pledge and tape it to your instrument. Each morning, before beginning your experiment, read it out loud so your labmates can hear. See you again next year.

Biosensor Pledge

1. I pledge to do my experiment right.
2. I pledge to report how I did my experiment.
3. I pledge to publish easily interpretable figures of my data that include replicates and an overlay of the fit to a simple model.
4. I pledge to report the binding constants and standard errors correctly.

BIOSENSOR VOCABULARY LIST

ancient school	using Scatchard plots to determine the equilibrium dissociation constant.
bulk-shift INcorrection	using the bulk-shift correction to mask complex data.
concentration-dependent aggregator	analyte that displays inexplicably large responses at high concentrations.
detergent-sensitive binder	analyte that becomes well-behaved upon the addition of detergent.
double referencing	subtracting the responses from a reference surface and buffer blanks.
Goldilock's responses	responses that are neither too big nor too small, but just right.
Hal-ing your results	publishing whatever the computer tells you.
hate constants	reported rate constants that do not describe the binding responses.
it might be mites	the idea that mites are the cause of all diseases.
k_a	association rate constant, with units defined as $M^{-1} s^{-1}$ by IUPAC IUBMB.
k_d	dissociation rate constant, with units defined as s^{-1} by IUPAC IUBMB.
K_D	equilibrium dissociation constant, with units defined as M by IUPAC IUBMB.
kinetic titration	testing several analyte concentrations in one binding cycle.
Michael Jackson data	data that are bad, bad really bad,—you know it.
Myszka's Razor	'Stop using a conformational-change model to fit your poor-quality data'.
non-stoichiometric binder	analytes that bind more than the predicted R_{max}.
off rate	slang term for the dissociation rate constant.
old school	anything before 1990, the year the first Biacore biosensor was released.
on rate	slang term for the association rate constant.
one-shot kinetics	testing several analyte concentrations in parallel.
pearls-on-a-string analysis	testing a fine dilution series of analyte concentrations.
promiscuous binder	poorly behaved analytes that produce unrealistic responses.
replicates	overlaid responses from repeated tests of the same analyte concentration.
SNL approach	short-'n-long kinetic analysis of stable complexes, in which the dissociation phase of only the highest analyte concentration is monitored for a long time.
SSS plotting	scaling-to-the-spikes syndrome.
Thpppfbt	sound signifying derision directed towards anyone claiming biosensor-determined binding parameters never match those from solution-based experiments.

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